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THE OXYGEN CONSUMPTION IN VITRO OF BRAIN CORTEX, KIDNEY, AND SKELETAL MUSCLE FROM ADRENALECTOMIZED RATS[1]

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The lowered basal metabolic rate of animals following the removal of the adrenal glands (1) is a striking and constant feature of the changes which take place in all of the species so far investigated. It has not been definitely established whether this reduction is *a*, a specific change resulting from the lack of the adrenal cortical hormone; *b*, a manifestation of an interrelation between the adrenal gland and one or more of the other endocrines known to secrete hormones having a calorigenic action under physiological conditions, or *c*, the indirect result of progressive circulatory and respiratory failure. If the metabolic rate of the whole animal is the sum of the metabolism of all of the tissues plus a certain minimum functional metabolism as suggested by Barcroft (2), then the *in vitro* respiration rate of tissues taken from adrenalectomized animals might yield some information which would help to separate direct and indirect effects of adrenal insufficiency. Either the failure of the direct action of the adrenal cortical hormone on tissue respiration or the secondary failure of action of some other hormone might be expected to affect the *in vitro* as well as the *in vivo* oxygen consumption. However, a lowered basal metabolic rate resulting largely from failure of the circulation, external respiration and other aspects of the minimal functional activities would not necessarily be associated with reduced rate of respiration *in vitro* of the tissues from the same animal.

Field, Belding and Martin (3) demonstrated that the summated tissue respiration in normal adult rats amounts to about sixty-six per cent of

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the mean basal metabolic rate, the largest fraction of the remainder being attributed to minimal functional activity. The present communication presents a survey of organ respiration in adrenalectomized animals as part of a study of the relation of summated organ metabolism to basal metabolic rate in adrenal insufficiency.

METHODS. The rats used in this experiment were young adult males and females of the Slonaker-Wistar strain. The range of weight was from 94 to 143 grams. Animals used for comparison were in almost all cases litter mates. For eight to ten days preceding the beginning of each experiment the animals were kept in a warmed room under the same conditions of temperature and alimentation as were to be encountered after adrenalectomy.

After this preliminary acclimatization the animals were bilaterally adrenalectomized under pentobarbital anesthesia (37 mgm. per kgm. intraperitoneally) through the lumbar approach. A second series of animals was subjected to similar acclimatization and surgical procedure except that the adrenal glands were left *in situ* after stripping the pedicle and dissecting the gland free of the kidney.

Two groups of ten animals each were adrenalectomized at a sufficient interval to permit sampling over the later portion of the average survival time for adrenalectomized animals in this colony. The series were arranged to begin tests on the fourth post-operative day, and to include duplicate organ samples from two or more animals over fourth to eighth post-operative days inclusive. The respiration rates of the organs of the adrenalectomized animals were compared with the rates for the same organs on the same days after operation in the dummy operation control series.

The technique for the preparation of tissues and the measurement of rate of oxygen consumption has been described in previous communications (3, 4). The only deviations from the previous procedure include changes in method of preparation of brain cortex and the obtaining of ratios of the wet weight of tissues to their dry weight. In place of free-hand cutting of cortical material with small scissors, we obtained thin slices of cortex by pressing a small fiber template down upon the surface of the brain and shaving off with a razor blade the cortical tissue which was caused to bulge through the opening in the template. In this way it was possible to obtain thin uniform slices of relatively larger area and thus restrict trauma to a smaller proportion of the tissue taken for use in the Warburg vessels.

The ratios of the wet weight to the dry weight of tissues were derived from samples of organs taken at the time the slices were prepared for measurement of oxygen consumption. The organs were prevented from drying during sampling by keeping them at room temperature in petri dishes

lined with filter paper moistened with Dickens-Greville (5) Ringer's solution.

The wet weight of organ slices was obtained by direct weighing on a torsion balance, some slices were transferred to crucibles and dried to constant weight and others were placed in the respirometer vessels.

RESULTS. 1. *Relative water content.* Table 1 presents the ratios of the wet weight to the dry weight of the organs studied in the adrenalectomized animals and the control (dummy operation) animals from the fourth to the eighth post-operative day inclusive.

The ratio becomes larger with time in the organs taken from the adrenalectomized animals. In the case of kidney and skeletal muscle the most striking change occurs sharply between the fifth and sixth day, while in

TABLE 1

Wet weight/dry weight ratios of brain, muscle and kidney

POST-OPERATIVE DAY	NUMBER OF ANIMALS		BRAIN		MUSCLE		KIDNEY	
	Controls	Adrenal-ectomized	Control	Adrenal-ectomized	Control	Adrenal-ectomized	Control	Adrenal-ectomized
4	2	2	5.59	5.13	4.61	4.49	5.09	4.55
5	2	4	5.85	4.81	4.91	5.13	4.45	5.56
6	2	4	5.12	5.23	4.56	5.67	4.82	5.23
7	2	4	5.78	5.56	4.83	5.18	5.05	5.32
8	2	2	5.05	5.97	4.67	5.46	4.97	5.66
Mean			5.48	5.34	4.72	5.19	4.88	5.26
Standard error			±0.17	±0.20	±0.07	±0.68	±0.12	±0.86
P			0.5000+*		0.0170		0.1276	

* Greater than 0.5000.

brain the change appears to be somewhat more gradual. The control animals show no significant change in the ratio throughout the time period. The mean of the ratios for brain over the entire period show no significant difference between the adrenalectomized animals and the controls. The difference observed in the muscle taken from the two series indicates that there is an increase in total water of this tissue. The same comparison for kidney indicates no significant change. It must be remembered that the amount of water present in the kidney as glomerular filtrate or formed urine is capable of influencing the wet to dry ratios to a marked extent. Adrenalectomized animals show a progressive reduction in urine volume, and it follows that one might expect the kidney of an animal with intact adrenal glands to contain a relatively larger proportion of water present as glomerular filtrate or as urine than the kidney of

an adrenalectomized animal. If the factor of urinary water content were equalized in the adrenalectomized and the control animals the ratios might then differ more widely in such a way that the difference would indicate a much larger proportion of tissue water in the kidney of the adrenalectomized animal. In the absence of such correction the difference observed may be deceptively low.

2. *Organ respiration on wet-weight basis.* a. *Brain.* The data presented in table 2 indicate that the respiratory rates of brain slices from the two groups are not significantly different. No tendency of the respiratory rate to decline with time in any given period of measurement was observed.

b. *Skeletal muscle.* It was found that the respiratory rate of skeletal muscle did not differ in the control and experimental animals until the seventh and eighth days after operation, at which time muscle from experimental animals showed a somewhat lower rate of oxygen consumption than corresponding muscles from the controls. The difference between the means for the entire period yields a P value of 0.3232 which is clearly not significant (7).

An analysis of the same results was made so as to separate the initial three days of the post-operative period studied from the last two days. In spite of the absence of a sufficient depression of respiration to account for a significant difference between the mean rates over the entire interval studied, it is demonstrated that the fall in respiratory rate of muscle during the last two days ($P = 0.0098$) is sufficient to justify the assumption that the difference is a real one.

c. *Kidney.* The difference in respiratory rate between kidney slices from control and adrenalectomized animals is more striking than in the case of muscle. Following the fifth day after operation the rate of kidney respiration is reduced by more than fifty per cent as compared with the controls. The magnitude of this decline during the last three days of the period is sufficient to account for a significant difference ($P = 0.0590$) in the mean rates over the entire five days studied.

Separate comparison of the first two and the last three days after operation reveals no difference during the fourth and fifth days, but the comparison of the latter portion yielded a P value of 0.0056, or about six chances in a thousand of such a difference's being encountered through errors of sampling.

3. *Organ respiration on dry-weight basis.* The increase in the ratio of the wet weight to the dry weight seen in the organs of adrenalectomized animals suggests that a part, at least, of the decline in respiratory rate observed when measurements were expressed on the wet-weight basis might be associated with the increased water content of the tissues.

When the same values for oxygen consumption are expressed on a dry-

weight basis, it is noted that the differences observed on the wet-weight basis have almost entirely disappeared. The only statistically significant difference (fewer than five chances in one hundred) is seen in the comparison of the respiratory rate of kidney during the last two days of the period. If the ratios of wet weight to dry weight for kidney tissue did

TABLE 2A

Q_{O_2} on wet-weight basis

POST-OPERATIVE DAY	CONTROLS				ADRENALECTOMIZED			
	Number of animals	Brain	Muscle	Kidney	Number of animals	Brain	Muscle	Kidney
4	2	1.81	0.58	3.86	2	2.00	0.50	3.73
5	2	1.70	0.54	3.27	4	1.80	0.57	3.50
6	2	1.72	0.43	3.49	4	1.85	0.56	2.71
7	2	2.04	0.54	4.11	4	1.85	0.40	2.21
8	2	1.78	0.55	3.60	2	1.78	0.37	2.30
Mean.....		1.81	0.53	3.66		1.86	0.48	2.89
S.E. X.....		±0.061	±0.025	±0.146		±0.038	±0.039	±0.311

* Q_{O_2} = cu. cm. O_2/gm./hr.

TABLE 2B

Q_{O_2} on dry-weight basis

POST-OPERATIVE DAY	CONTROLS				ADRENALECTOMIZED			
	Number of animals	Brain	Muscle	Kidney	Number of animals	Brain	Muscle	Kidney
4	2	10.1	2.7	19.6	2	11.0	2.4	17.8
5	2	10.0	2.6	14.5	4	9.2	2.6	15.9
6	2	8.8	2.0	15.1	4	8.9	2.9	15.1
7	2	11.8	2.6	20.7	4	9.7	2.3	11.6
8	2	9.0	2.5	17.9	2	9.9	1.9	12.3
Mean.....		9.9	2.5	17.6		9.7	2.4	14.5
S.E. X.....		±0.54	±0.13	±1.22		±0.33	±0.15	±1.18

not suffer from the error introduced by the difference in urinary water the values should yield an even smaller difference.

It is interesting to note that in brain, in which no difference in relative water content was noted, there appears no "decrease" of respiration when the oxygen consumption is expressed on the wet-weight basis or the dry-weight basis over the entire period of insufficiency studied. This observation is in substantial agreement with Tipton's recent report (8).

DISCUSSION. The decline of basal metabolic rate, expressed on a calculated surface area basis, of adrenalectomized animals begins usually a little more than half way through the survival period of insufficiency (1) and falls from 20 per cent to 50 per cent below the pre-operative value.

The observed lowering of respiration in both kidney and muscle calculated on the wet-weight basis is in rough agreement with the magnitude of the change reported for the intact animal and is not strikingly different in its time course. However, when the *in vitro* respiration is calculated on the basis of dry weight, the magnitude of the difference between the experimental group and the controls becomes insignificant with the exception of that of kidney, in which the observed difference would be encountered only three times in one hundred in random sampling of a series in which no real difference existed.

This observation indicates *a*, that the lowered basal consumption seen in adrenalectomized animals is not entirely the result of failure of minimal functional activity, and *b*, that the altered water distribution can account for the change observed in the *in vitro* respiration of brain and muscle, and in part for the change in respiration of kidney when the results are expressed on the wet-weight basis.

The prime importance of relative water content in determining the direction of change in respiration described is entirely consistent with the cellular hydration reported in adrenal insufficiency (9, 10, 11) and in similar electrolyte imbalances induced by intraperitoneal injections of glucose (12), or direct alterations of the sodium and chloride concentration of the extracellular fluid (13).

Under the conditions of these experiments, the influence of secondary factors, consequent upon water and electrolyte changes is markedly reduced. After the transfer of the organ slices to Dickens-Greville Ringer's solution, a large portion of the water weighed out with the sample becomes metabolically ineffective *in vitro* because it is no longer in the cell.

Field, Belding and Martin (4) in discussing the absence of significant difference between variability of Q_{O_2} on wet and dry bases state "an important theoretical consequence is that tissue water content is related as closely, in a statistical sense, to the factors determining Q_{O_2} as is the miscellaneous aggregate of substances making up dry weight." The findings in the present experiment emphasize the validity of this claim. It is only required to point out the necessity of restricting the use of the wet-weight basis to situation in which there is no possibility of a shift of water into the tissues and further, the desirability of employing a medium in the *in vitro* situation which will imitate as closely as possible the environment of the cells *in vivo*.

The decrease in rate of respiration of kidney tissue which was demonstrated on both the wet and dry weight bases is interesting in connection

with the important rôle played by this organ in the secondary changes of adrenal insufficiency. Since the respiratory rate of kidney is the highest per unit weight of any tissue in the body it is not remarkable that interference with oxidative metabolism through a defect in phosphorylation (15) or in other ways (6) would be manifested first and most profoundly in the kidney. In tissues having a slower respiration the result of interference with metabolism in the same way may be so small in magnitude as to be obscured by the consequences of the changes in electrolyte pattern induced. It may be suggested that the only way clearly to demonstrate the direct effect of lack of adrenal cortical hormone on the metabolism of tissues other than the kidney would be to protect them from intercurrent injury and secondary influences until sufficient time has elapsed for the specific deficiency to be manifested.

SUMMARY

1. The *in vitro* respiratory rates and the ratios of wet to dry weight of skeletal muscle, kidney, and brain from adrenalectomized rats and control animals subjected to a dummy operation of equal severity have been compared over the period from the fourth through the eighth day following operation.

2. Increases in water were found in muscle from adrenalectomized animals. The changes in the kidney are variable and those in brain insignificant.

3. The decrease in respiratory rate expressed on a wet-weight basis which was noted in kidney after the fifth day following adrenalectomy persisted but was found to be smaller in magnitude when the same rates were expressed per gram dry tissue. The differences observed in muscle respiratory rate disappeared when the rates were calculated on the dry-weight basis.

4. The respiration of brain does not change significantly.

5. It is suggested that:

a. The significant reduction of rate of respiration, based on the dry weight, of kidney from adrenalectomized animals may be causally related to the high rate of respiration of this organ.

b. The use of the wet weight as a basis for expressing results of *in vitro* studies of organ metabolism should be restricted to situations where there is no possibility of a shift of water from one "compartment" of the tissues to another.

c. A clear demonstration of the direct effect of absence of the adrenal cortical hormone on the metabolism of tissues other than the kidney is possible only under conditions which protect those tissues from intercurrent injury and secondary effects of deficiency until sufficient time has elapsed for the specific deficiency to be manifested.

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REFERENCES

(1) GROLLMAN, A. The adrenals. Williams & Wilkins Co., Baltimore, 1936.
(2) BARCROFT, J. Ergebn. d. Physiol. **7:** 699, 1908.
(3) FIELD, J., 2ND, H. S. BELDING AND A. W. MARTIN. J. Cell and Comp. Physiol. **14:** 143, 1939.
(4) FIELD, J., 2ND, H. S. BELDING AND A. W. MARTIN. Proc. Soc. Exper. Biol. and Med. **40:** 565, 1939.
(5) DICKENS, F. AND D. G. GREVILLE. Biochem. J. **27:** 832, 1933.
(6) HARROP, G. A. Bull. Johns Hopkins Hosp. **59:** 11, 1936.
(7) FISHER, R. A. Statistical methods for research workers. 6th ed. Oliver and Boyd, Edinburgh, 1936.
(8) TIPTON, S. R. This Journal **127:** 710, 1939.
(9) LOEB, R. F., D. W. ATCHLEY, E. M. BENEDICT AND J. LELAND. J. Exper. Med. **57:** 775, 1933.
(10) HARROP, G. A., A. WEINSTEIN, L. J. SOFFER AND J. H. TRESCHER. J. Exper. Med. **58:** 1, 1933.
(11) HARROP, G. A., L. J. SOFFER, R. ELLSWORTH AND J. H. TRESCHER. J. Exper. Med. **58:** 17, 1933.
(12) GILMAN, A. This Journal **108:** 662, 1934.
(13) DARROW, D. C. AND H. YANNET. J. Clin. Investigation **14:** 266, 1935.
(14) PETERS, J. P. Body water. Chas. C. Thomas, Springfield, Ill., 1935.
(15) VERZÁR, F. AND L. LASZT. Pflüger's Arch. **237:** 476, 1936.

THE ACTION POTENTIALS OF THE SQUID EYE

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In the cephalopod eye, containing only sensory cells, the electroretinogram (ERG) has been found to consist of a single sustained potential (Beck, 1899; Piper, 1904; Fröhlich, 1913). Brücke and Garten (1907) and Piper (1911), considering the inversion of the vertebrate retina, identified this monophasic action potential on an electrical basis with the "positive" action potential (i.e., the *b*-wave or component PII; Granit, 1933) of the vertebrate eye. The analogy thus would require PII to be localized to the receptorial layer of the vertebrate retina, a suggestion already made by Kühne and Steiner (1881). However, in view of the complex structure of the vertebrate eye, an attempt to identify components of the vertebrate ERG with the receptorial action potential of the cephalopod eye should be made not only on an electrical basis (cf. Therman, 1938; Granit and Therman, 1938) but at the same time also by a comparison of the effects of different biological agents.

Besides a general investigation of the nature of the squid ERG, the present paper deals with a study of the effects of glucose, potassium, atropine and adrenaline, as well as oxygen, on the ERG of the cephalopod eye (*Loligo pealii*).

It is worthy of mention that earlier electrophysiological work on the cephalopod eye has been done chiefly on *Eledone moschata*, and Fröhlich (1913) reports that he did not find the eye of *Loligo* as useful as that of *Eledone*. However, the anatomical structure of the eye in both species is essentially the same (Grenacher, 1886; cf. also Hess, 1905; Hanström, 1928).

METHOD. The squids were kept in running sea-water in a dimly illuminated basin. A number of experiments were also carried out on squids kept in light or in complete darkness. A 200-W electric bulb was used as a source for the stimulating light, which passed through a condenser lens and a water-filter. The strongest intensity used (I/1) was 300 Lux. By means of Wratten neutral-tint filters the intensity could be

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varied between I/1 and I/10,000. The light was thrown into the whole eye by a mirror above the eye-holder. The time and length of exposure was controlled by a photographic shutter. The length of the exposure in routine experiments was 0.25 to 0.5 sec. and the interval between each stimulation 2 to 5 min.

As several electrodes were placed at the same time on the eye, it was found convenient to use plain chlorided silver wires. Photo-electric effects were constantly watched for and were kept below 10 μV.

The recording equipment consisted of two condenser-coupled amplifiers in connection with a two-channel ink-writer, thus enabling simultaneous records to be taken from two different points. The time constant used (halfway down in 0.7 sec.) was long enough to reveal the essential features of the ERG of short exposures. However, it did not permit observations of a slow process like the PI component (*c*-wave) in the vertebrate eye. The frequency characteristic of the recording system should be considered as also limiting the possibilities of recording action potentials of a duration comparable to axon spikes.

Results. The electrical activity of more than 80 eyes from different squids (*Loligo pealii*) has been studied. For a general schematic view of the electrode placements as well as the relative size and polarity of the responses see figure 1 and legend.

On the whole no pronounced electrical activity could be revealed by any combination of electrodes E_3, E_4, E_5 and E_6. A small positive action potential was often recorded at E_3. Its size fluctuated with the size of the positive deflection recorded with E_2 (see below) and was also dependent on the distance to the fundus of the eye, being smaller the longer the distance. Records taken with E_5 connected to E_6 gave in a few cases a faint sustained negative potential. In one single case it remained for more than one hour and was of the order of 10 to 40 μV (see fig. 4), while in the other cases it soon disappeared. This rare occurrence of a weak electrical activity in the ganglion or its optic nerve is in sharp contrast to the bigger and easily obtained potentials from the eye-ball.

The ERG. It was soon found that in the case of a dark-adapted animal the weak light (red or white) necessary for a successful enucleation was strong enough to depress the size of the action potential. The subsequent enhancement is comparable to the effect of the regeneration of visual purple on the ERG of the frog's retina. This resemblance is also emphasized by the occurrence of a red color in the squid retina after being kept in darkness for one-half to one hour. It is found that the enhancement of the potential is facilitated when the eye is opened (cf. Granit and Munsterhjelm, 1937).

Provided that moderate intensities are used, a monophasic, sustained, positive action potential is recorded when one electrode, E, is placed on

the surface of an intact eye and the second electrode, E_6, as reference point, on the ganglion (cf. fig. 1, A). The shape and size of the action potential does not depend on whether the second lead is on the ganglion or *Nervus opticus*, nor will an appreciable change occur when the *Nervuli optici* are crushed. The size of the potential, however, is largely de-

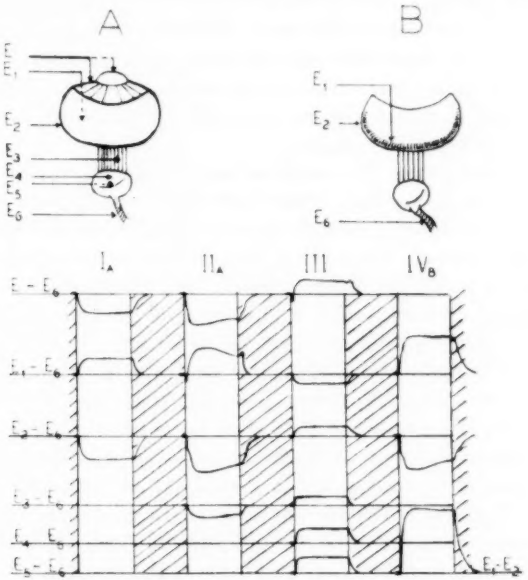

Fig. 1. Schematic view of the electrode placement in an intact (A) and an opened (B) eye as well as corresponding records obtained with indicated electrode combinations. Of these electrodes (corresponding to A) all but E_1 and E_5 were surface electrodes. E_1 and E_5 consisted of thin chlorided silver wires insulated to their tip and were inserted into the tissue. Upward deflection indicates negativity at the low-numbered electrode. The intensity of the stimulus in group I_A and IV_B was I/100, in group II_B I/10. Time of exposures, 0.25 sec.

Group III contains records representing observations of "atypical" action potentials assembled from different experiments. For their relationship to the regularly obtained deflections reproduced in the groups I_A, II_A and IV_B see text.

pendent on the placement of the eye-electrode. The biggest response is often obtained from the scleral part, the smallest, from the lens.

If strong intensities are used (e.g., I/10; cf. fig. 1, II_A, $E - E_6$) and the eye has been kept in darkness for some time, the positive action potential recorded is not smooth. At both "on" and "off" there are indications of an interfering action potential of opposite electrical sign. However, these irregularities are more commonly seen if the eye has been damaged

in some way. In fact, pure negative action potentials may be obtained if the "active" electrode is in contact with the ocular fluid (see fig. 1, A, $E_1 - E_6$; III, $E - E_6$). These results indicating the presence of two action potentials of opposite sign with regard to a common reference point on the optic ganglion were confirmed and more clearly brought out by using opened eyes (fig. 1, B). With regard to the reference electrode, E_6, the electrode on the receptorial layer, E_1, revealed a negative action potential while E_2 at approximately the same time became positive. The relative size of these two action potentials may be changed by certain factors such as state of adaptation, strength of stimulation, effect of drugs, or impaired condition of the eye.

In light-adapted eyes the negative action potential (at E_1) is usually lacking and only a positive action potential of moderate size may be recorded from the outer surface of the eye, E_2, or from the exposed peripheral side of the retina. However, if the eye is kept in darkness a negative action potential at E_1 gradually appears. At the same time the positive action potential increases also. This gradual increase of both action potentials continues during the next 30 to 60 min. but follows different curves (fig. 2). Again, long exposures or frequent stimulation exhaust more rapidly the negative action potential than the positive one. At the end of the enhancement of the action potentials, the records obtained with strong intensities and an electrode inside the eye, E_1, usually show a positive deflection preceding the negative action potential (fig. 1, II_A; $E_1 - E_6$). This positive deflection may persist as a monophasic positive action potential irrespective of the disappearance of the negative action potential and show the same characteristics as the positive action potential recorded at E_2. However, the size of such positive deflections recorded at E_1 are always much smaller than the corresponding potential at E_2. A similar "spread" of the negative action potential is indicated by the above mentioned irregularities at "on" and "off" of the positive surface potential, E_2. The positive action potential, E_2, has never been seen preceded by a negative deflection.

The positive action potential responds with deflections of greater differences in size within a certain range of intensities than the negative one. The following figures were obtained simultaneously for the negative and positive deflections from the same eye stimulated with two different intensities; I/100: pos. 20 μV, neg. 20 μV; I/10: pos. 70 μV, neg. 45 μV. The positive action potential thus was increased 3.5 times, while the negative was increased only 2 times.

If the electrical activity of the retina is recorded between the electrodes E_1 and E_2, instead of using a common reference electrode, E_6, a smooth action potential is obtained. Its polarity *always* indicates negativity at the end of the receptors (i.e., *Membrana limitans*) and positivity at the

base (fig. 1, IV_B, $E_1 - E_2$). The size of this deflection is always bigger than the "individually" recorded potentials (IV_B) and may be even bigger than their sum.

Effect of chemical agents. The effect of different chemical agents emphasizes the relative differences between the negative and positive action potentials. Such differences occur both with respect to the time of onset as well as to the degree of the effects.

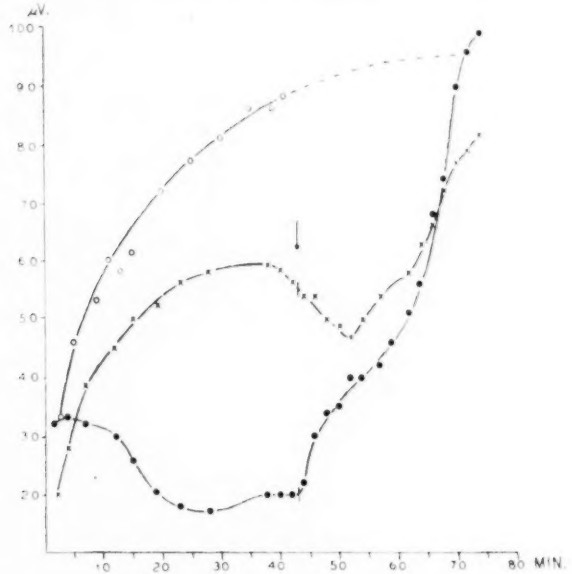

Fig. 2. Eye previously light-adapted. The curves show subsequent increase of the negative (dots) and positive (crosses) action potentials. Glucose was given at the moment indicated by arrow. For comparison a curve is drawn of a "normally" increasing negative action potential (circles) obtained from another animal. Note correspondence in the starting and end points of the two curves (dots and circles).

Glucose (isotonic with sea-water) enhances both the negative and the positive deflections but the maximal effect occurs at different times and to a different extent, as is seen in figures 2 and 3. Both potentials may maintain their final size for several hours. A second drop of glucose is without any appreciable effect. With regard to the negative action potential the glucose effect is most striking in a previously light-adapted eye which has been kept in darkness without showing any significant increase of this potential. This is shown in figure 2, where the regeneration of the positive (crosses) and negative (dots) action potential is seen before and

after a glucose treatment. For comparison a regeneration curve (circles) of the negative action potential in another eye under optimal conditions (without glucose) is drawn in the same diagram.

If a light-adapted eye is filled with glucose immediately after the enucleation, a smooth increase of both positive and negative action potentials is obtained. The rate of increase as well as the final size of the deflections is directly comparable with that obtained in the best experiments without a previous treatment with glucose.

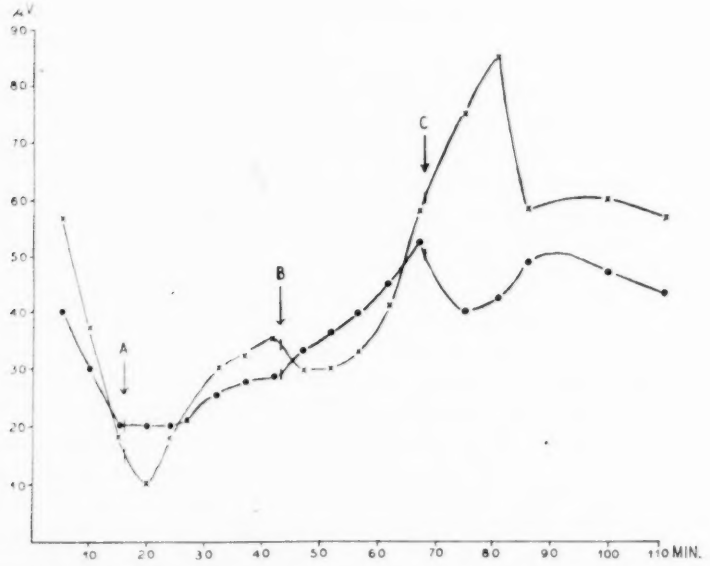

Fig. 3. The effects on the negative (dots) and the positive (crosses) action potentials by successive treatments of a previously light-adapted eye with oxygen (at *A*), glucose (at *B*) and adrenaline (at *C*).

In another series of experiments, a continuous stream of pure oxygen was led directly into the dark-box and the favorable effect obtained resembles that of glucose. However, in most of the cases the positive action potential is affected far more than the negative whereas the opposite is true for the glucose effect. A subsequent treatment with glucose is thus usually effective in increasing the negative action potential.

The solvent for the different potassium concentrations, ranging between 0.01 and 10 per cent, has been mostly glucose. After the subsidence of the glucose effect a potassium-glucose solution was added. Generally both the negative and the positive action potentials show a great resistance

to treatments with potassium solutions. Concentrations below 0.5 per cent were hardly effective at all. Clearer effects were obtained only with stronger potassium solutions. A facilitation of both the positive and negative action potentials (particularly the positive) may be obtained with 0.5 to 2 per cent potassium solutions (fig. 4). With still stronger potassium solutions (2 to 10 per cent) a decrease of both action potentials

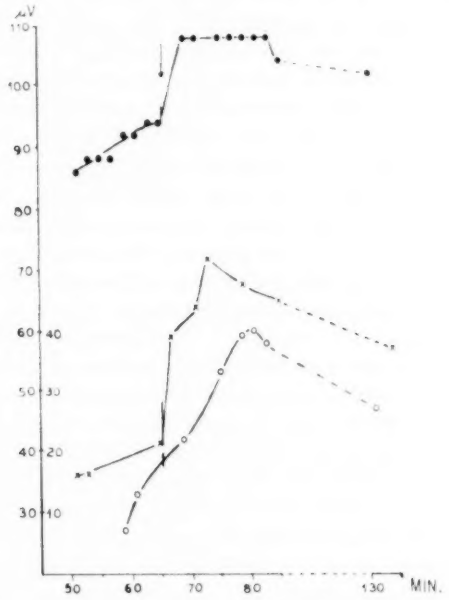

Fig. 4. The effect of potassium on the retinal action currents and the ganglionic action potential. Negative action potential (electrodes $E_1 - E_6$, fig. 1; IV_B) = dots; positive action potential ($E_2 - E_6$) = crosses; ganglionic action potential ($E_5 - E_6$, group III) = circles. The eye, previously treated with glucose, was filled with a 1 per cent KCl solution (dissolved in glucose) at the moment indicated by arrow. The size of the ganglionic action potential corresponds to the inside scale.

is generally observed, but, while the negative deflection declines more and disappears from the record, the positive one, though decreased, still may remain.

The effect of adrenaline (dissolved in glucose) consisted of a slow depression of both action potentials, as seen in figure 3. This depressant action does not affect appreciably the shape of the deflections.

The effect of atropine (1 per cent dissolved in glucose) resembles the effect of adrenaline. A marked facilitating effect of atropine on either action potential has not been observed.

Discussion. The reason that a ganglion action potential has been so rarely seen, as well as its early disappearance compared with the resistant action potentials of the eye, may be found in structural and functional differences between receptors and ganglion cells, the latter being more susceptible to removal from the natural environment. The small positive action potential sometimes recorded from the *Nervuli optici* is obviously due to an electrotonic spread (cf. Eccles, 1935) of the main action potential originated in the retina.

Considering the fact that during illumination two action potentials of opposite electrical sign (with regard to a reference point) can be recorded from opposite sides of the receptorial layer of the squid's retina, the question arises whether these potentials actually represent bio-electrical signs of two different processes, or whether the difference in polarity is merely due to the electrical pathways involved and we thus are recording one and the same process. On account of general observations on the effect of a proceeding dark-adaptation and intensity-variation it can be tentatively concluded that the two action potentials are two different processes. This suggestion is supported by the differential effects of glucose, oxygen, potassium, adrenaline and atropine. If the two action potentials were due to a common process and thus merely signs of a "dipole" electrical system it would be reasonable to expect a closer agreement in their mutual variations. Furthermore, the presence of two retinal action potentials in the squid eye may easily explain the old differences in opinion about the polarity of the cephalopod ERG (cf. Beck, 1899; Piper, 1904).

From the electrical point of view, it may be emphasized that the potential distribution found (positivity at the base and negativity at the end of the receptors) never is completely reversed, and that the two action potentials add and give a maximal smooth ERG if the electrodes are on opposite sides of the retina. The fact that a small negative deflection may, in the absence of the positive action potential, be recorded from the outside of the eye,—or *vice versa* a small positive deflection may, in the absence of a negative action potential, be recorded from the inside of the eye (cf. fig. 1, III)—seems also to indicate that the two action potentials are due to different processes. With regard to the placement of the electrodes with a common reference electrode on the ganglion, the positive action potential should be expected to counteract the recording of the negative action potential. The "true" size of the negative action current can be approached only by subtracting the positive deflection from the combined electrical activity obtained in an electrode combination of $E_1 - E_2$ (fig. 1, IV_B). According to this view a main increase of the positive action potential should also be revealed as a decrease in the recorded negative deflection (provided that the actual size of the latter

is unchanged). Such opposite changes are commonly seen and demonstrated in figures 2 and 3. It is also interesting to note that the positive deflection seems to be generated faster than the negative as is indicated by the appearance of a positive "notch" preceding the negative action potential (cf. fig. 1, II_A, $E_1 - E_6$). A similar positive "notch" preceding the main retinal action potential of *Eledone moschata* has been reported by Fröhlich (1913).

In his elaborate study of the histology of the cephalopod retina, Grenacher (1886) has pointed out that the cephalopod retina possesses only rods and that the picture reveals essentially the same details as are found in rods of amphibians and mammals. Three parts of the rod are distinguishable: the outer and inner segments and the nuclear part. It is generally believed that these parts are structural differences within a single cell (cf. Detwiler, 1938), although a different opinion exists (cf. Moroff, 1922). The inner segments have been assumed by Kühne and Steiner (1880) to possess electrical activity during illumination, whereas Garten (1907) in this respect emphasized the importance of the outer segments. However, at that period the suggestion referred only to the *b*-wave (PII) of the frog's ERG.

In general the various agents used in this investigation affect both action potentials of the squid eye in the same direction. Neither agent has been shown to elicit a clear enhancement of one action potential while the other is being depressed. In the frog eye weak concentrations of a potassium solution are sufficient to depress or remove the PII component while PIII even by stronger solutions is unaltered or slightly increased (Therman, 1938). In the squid eye both the negative and positive action potentials are strikingly resistant to a treatment with potassium and may even show an increase in size. The specific effects of adrenaline and atropine on the PII component of the frog ERG (Therman, 1938) have not been observed in the squid eye. These results inevitably suggest an identification of both the negative and the positive action potential of the squid eye with the PIII component of the frog eye. Irrespective of the close resemblance in shape of the combined action potentials of the squid eye ($E_1 - E_2$, fig. 1, IV_B) and the PIII component in the vertebrate eye, it remains to be seen whether PIII also involves two electrophysiological processes.

Electrophysiologically PIII is the first response to be seen in a vertebrate eye at the moment of light-stimulation, but, also, ontogenetically this component is the first to occur in a developing rat's retina (Keeler *et al.*, 1928). This elementary character of the PIII component is emphasized by the identification of PIII with the receptorial action potentials of the squid eye.

SUMMARY

The electrical response of the squid eye (*Loligo pealii*) to illumination has been recorded from different parts of the eye-bulb and ganglion by means of two condenser coupled amplifiers connected to a two-channel ink-writer.

A faint, subsiding action potential has been recorded occasionally from the optic ganglion.

Two monophasic, sustained action potentials of opposite electrical sign (with respect to a common reference electrode on the optic nerve) have been recorded from the retina. A negative deflection is recorded from the end part and a positive from the basal part of the receptors. In bipolar recording with electrodes on opposite sides of the retina the potential obtained is approximately the sum of the two action currents and the polarity indicates negativity at the end and positivity at the base of the receptors.

The two action potentials are in general affected by a progressive dark-adaptation, oxygen, glucose, potassium, adrenaline and atropine in the same direction but to a different degree and at different times. Both the negative and the positive action currents (and thus their combined electrical activity) resemble in shape and reaction the PIII component of the vertebrate eye.

The electrical recording system, designed by Mr. A. M. Grass, was kindly put at my disposal by the department of Physiology of the Harvard Medical School, for which I am greatly indebted to Dr. Hallowell Davis.

REFERENCES

BECK, H. Pflüger's Arch. **78:** 129, 1899.
BRÜCKE, E. T. v. AND S. GARTEN. Pflüger's Arch. **120:** 290, 1907.
DETWILER, S. R. Yale J. Biol. and Med. **10:** 485, 1938.
ECCLES, J. C. J. Physiol. **85:** 179, 1935.
FRÖHLICH, F. W. Ztsch. Sinnesphysiol. **48:** 28, 1913.
GARTEN, S. Handb. ges. Augenheilk. (Graefe-Saemisch) **3:** chap. 12, 1–250, 1907.
GRANIT, R. J. Physiol. **77:** 207, 1933.
GRANIT, R. AND A. MUNSTERHJELM. J. Physiol. **88:** 436, 1937.
GRANIT, R. AND P. O. THERMAN. J. Physiol. **93:** 9P, 1938.
GRENACHER, H. Abh. der Natruforsch. Gesellsch. zur Halle **16:** 207, 1886.
HANSTRÖM, B. Vergleichende Anatomie des Nervensystems der Wirbellosen Tiere. Berlin, Springer, 1928.
HESS, C. Pflüger's Arch. **109:** 393, 1905.
KEELER, C. E., E. SUTCLIFFE AND E. L. CHAFFEE. Proc. Nat. Acad. Sci. **14:** 811, 1928.
KÜHNE, W. AND J. STEINER. Untersuch. Physiol. Inst. Heidelberg **3:** 327, 1880; **4:** 64, 1881.
MOROFF, T. Anat. Anz. **55:** 316, 1922.
PIPER, H. Arch. Physiol. 453, 1904; 85, 1911.
THERMAN, P. O. Acta Soc. Scient. Fenn., Nova Ser. B, **2:** 1, 1938.

THE GLUCOSE UTILIZATION OF HEPATECTOMIZED DIABETIC RABBITS

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The determination of the glucose consumption rate of the extra hepatic tissues of the depancreatized animal is of first importance in solving the problem of diabetes. Such a determination also throws light on the question of conversion of fat to carbohydrate in this disease. One must remove the liver to carry out a glucose balance in any animal since this organ at all times is adding an indeterminate amount of sugar to the blood. Removal of the gastro-intestinal tract is also necessary if the animal has been recently fed since then absorption of glucose from the tract is a complicating factor.

The principle of the method is simple. Diabetes is produced by pancreatectomy and some time later the liver is removed and one determines the rate at which glucose must be injected intravenously to maintain the blood sugar constant at the preoperative level. The amount of sugar excreted by the kidneys during the determination must be subtracted from the amount injected to give the net injection rate. One infers then that the tissues are removing the glucose from the blood stream at this net rate and one assumes that the tissues had been behaving in the same way prior to the operative removal of the liver.

Mann and Magath (3) showed that the blood sugar of the diabetic dog fell after hepatectomy, but did not measure the glucose utilization rate. Yater et al. (4) made such a quantitative study. They injected glucose at a rate sufficient to maintain the blood sugar constant and found that the normal hepatectomized dog used glucose at the rate of 250 mgm. per kilo per hour and the hepatectomized diabetic dog at 190 mgm. per kilo per hour. There are definite objections to the conditions of their experiments. The kidneys were removed; this has been shown to increase glucose disappearance (1). No mention is made as to whether the animals were fasted or not; this too has a bearing on the results (5). Furthermore these authors state as does Mann (6) that the depancreatized dog does badly after hepatectomy and rapidly becomes moribund. Anyone familar with glucose utilization determinations in hepatectomized animals is

aware of the marked effect that poor general condition may have on the rate of glucose disappearance, usually increasing it. In the final breakdown of the animal, commonly referred to as "the second stage after hepatectomy", the glucose requirement is markedly increased (7). If the diabetic condition causes the onset of the second stage to come on prematurely in dogs, this would tend to cause a high glucose requirement. Besides this, we have observed that the development of any symptoms indicating that the physiological processes of the body are not altogether normal is often accompanied by an increase in glucose need. This is illustrated by the results in a group of hepatectomized and eviscerated rabbits which we had eliminated from other series and not reported,

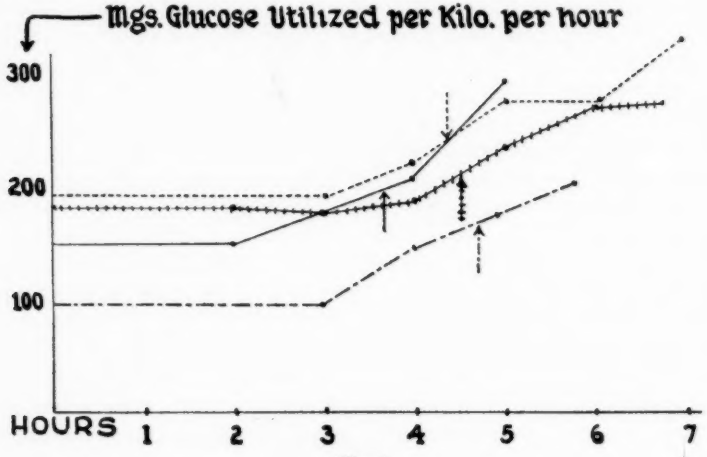

Fig. 1

because the condition of the animals was unsatisfactory and not in keeping with the requirements which we have stipulated for such experiments (5). For these animals the note "head drooping" (indicating weakness or poor righting reflexes) was put into the record by the observer without his having had any regard to the glucose utilization rate at the time. In figure 1 are depicted the results in these animals showing the increase in glucose utilization which develops after a few hours. The arrows indicate the time when the note was made regarding the condition of the animal (head drooping). It is quite obvious that the glucose utilization increases as the condition of the animal becomes worse. These results are to be compared with those of hepatectomized, and of eviscerated rabbits that remain in good shape and that have practically a constant utilization throughout (1, 8).

It appeared to us that much of the trouble experienced by previous workers resulted from their use of dogs. We decided to substitute rabbits for this work. This animal becomes diabetic after pancreatectomy (9), and one is able to study such animals after evisceration, i.e., removal of the liver and intestinal tract. They withstand this procedure remarkably well. They make good recovery from operation, sit up normally, the kidneys excrete urine and they survive for many hours, and so satisfy the requirements which we have demanded in this laboratory for the glucose utilization rate determination after hepatectomy or evisceration.

METHOD. Pancreatectomy was performed by the method of Greeley (9) and at this time a silk ligature was placed around the inferior vena cava just above the right renal vein which constricted this vein to approximately 1.8 mm. This was done as a necessary preliminary preparation for eviscer-

TABLE 1

NUMBER	SEX	WEIGHT AFTER EVISCERATION	DURATION OF DIABETES	BLOOD SUGAR BEFORE EVISCERATION	GLYCOSURIA AFTER EVISCERATION FOR 6 HOUR PERIOD	UTILIZATION OF GLUCOSE PER KILO HOUR AFTER EVISCERATION (CORRECTED FOR GLYCOSURIA)	O_2 UTILIZATION PER KILO PER HOUR	PREOPERATIVE NUTRITIONAL STATE
		grams	*days*	*mgm. per cent*	*grams*	*grams*	*cc.*	
1	F	1350	7	380	0.042	0.217	520	Fed
2	M	1075	4	320	0.152	0.201	351	Fed
3	M	1225	4	322	0.252	0.149	440	Fed
4	M	1220	9	380	0.018	0.053	216	Fed
5	F	1300	8	400	0.222	0.115	540	Fasted 2 days

ation (8). About two weeks later the remaining pancreas in the duodenal loop was removed. Complete evisceration, except for the urogenital system, was performed from 3 to 22 days after pancreatectomy.

As shown in table 1 some animals were fed up to the time of the evisceration and others were fasted for two or three days. Evisceration was done under ether anesthesia, the bladder washed and a catheter left in place in order to measure the glucose excreted during any given period. The blood sugar found immediately before evisceration was maintained after evisceration by the intermittent intravenous injection of glucose every thirty or sixty minutes. Glucose utilization was determined by subtracting the amount of sugar lost in the urine from the glucose administered intravenously to maintain the constant blood sugar level. Oxygen consumption was determined by connecting the tracheal cannula, which had been inserted at the time of evisceration, to a closed circuit

which contained a soda lime cartridge for the removal of CO_2, and a pump to insure circulation of air, and a small Krogh spirometer to register the oxygen used. The temperature of the animal was maintained during evisceration by an ordinary electric heating pad. Kidney function was tested by the intravenous injection of 1 cc. of 0.1 per cent phenol red as previously described (5). Eleven animals were carried out in this way.

RESULTS. Any animals that did not appear absolutely normal after operation or did not have functioning kidneys were eliminated. The results are given in table 1 for animals that satisfied all requirements and in whom the glucose utilization rate was constant during the period of

TABLE 2

NUMBER	SEX	WEIGHT AFTER EVISCERATION	DURATION OF DIABETES	BLOOD SUGAR BEFORE EVISCERATION	UTILIZATION OF GLUCOSE PER KILO PER HOUR AFTER EVISCERATION (CORRECTED FOR GLYCOSURIA)	GLYCOSURIA AFTER EVISCERATION FOR 6 HOUR PERIOD	O_2 UTILIZATION PER KILO PER HOUR	PREOPERATIVE NUTRITIONAL STATE
		grams	*days*	*mgm. per cent*	*grams*	*grams*	*cc.*	
1	F	1150	9	500	0.224* 0.324†	0.041	422	Fed
2		1370	7	110	0.109* 0.360†	0.00	354	Fasted 2 days non-fasting blood sugar 331
3	M	1175	22	204	0.172* 0.383†	0.00	413	Fasted 3 days non-fasting blood sugar 364
4	M	1675	13	160	0.119* 0.348†	0.00	358	Fasted 3 days non-fasting blood sugar 470
5	M	1150	16	100	0.174* 0.348†	0.00	234	Fasted 2 days non-fasting blood sugar 400

* First 3 hours.

† Last 3 hours.

determination. There was a group of animals that satisfied the requirements above but in which the rate of glucose utilization increased progressively the longer the determination was carried. We have noted this type of behavior before in eviscerated rabbits (5) although it is not usual. Ordinarily the rate does not change significantly. These animals are given separately (table 2) as it appears to us that it would be a mistake to report the average figure for the glucose utilization rate when this has changed during the run. For these animals therefore we give both the rate for the first 3 hours of the run and that for the later period when it was much higher. It seems most likely that some unknown factor begins to operate as time goes on in these animals to increase their rates and that the rate found soon after operation is closest to the glucose utilization of the tissues before operation.

Discussion. We have not made muscle glycogen determinations in order to correct our utilization rates for the changes that might occur in muscle glycogen, as is done by Soskin and Levine (10). In hepatectomized animals the muscle glycogen practically always stays level or goes down. In doing glucose balance studies, if there is a rise in muscle glycogen it is proper that one should correct for the increase since it would probably have come from glucose. But it does not follow that one must make a similar correction for a drop in muscle glycogen when the liver is absent, for in such preparations there is no way for conversion of muscle glycogen to utilizable glucose. When muscle glycogen disappears from such animals it does not go to "spare" the glucose that has to be injected to keep the blood sugar constant; it seems chiefly to be changed to lactic acid which either accumulates in the body or is burned as such.

Before anything else we would want to stress the need of caution in interpretation of results obtained from hepatectomized diabetic animals. Such animals sometimes show large changes in glucose utilization and one must guard against making too hasty conclusions from calculations based on such results. Young (11) on the figures from one such animal attempts to prove that fat must be changed to carbohydrate. If one wished to select certain results in our series one could make similar conclusions. The opposite could easily be proven with another selection.

Our results support the contention that the tissues of the fasting diabetic animal use glucose at the same rate as the normal. Indeed the results of some of our animals would indicate that the rate is higher in the diabetics, but there is the objection to these animals that their rates were not steady after operation, but increased after a time. No animal having a steady rate showed a higher rate than normal animals. If we accept the view that the rates obtained during the first 3 hours after operation are the closest to the rates of sugar utilization by the tissues before operation, we obtain an average figure of 138 mgm. per kilo per hour. To supply such an amount of glucose to the tissues would not necessitate a conversion of fat to glucose. The fasting rabbit excretes about 25 mgm. of nitrogen per kilo per hour and if we accept a D:N ratio of 6 (12) this amount of glucose could be supplied by the conversion of the protein to glucose.

We confirm the findings of Mann (6) and of Yater et al. (4) that diabetic tissues can and do utilize glucose. In addition we can conclude that quantitatively the tissues of the fasted eviscerated diabetic rabbit utilize glucose at the same rate as the normal fasted hepatectomized rabbit which is 125 mgm. per kilo per hour (13). The simple hepatectomized rabbit has his pancreas intact and there is no reason to suppose that it is not functioning in a normal manner. We may conclude then that the presence or absence of the pancreas has no effect on the rate of oxidation of glucose by the tissues of the fasting animal. Our results do not bear on the problem

of glucose utilization as a source of muscle energy in the normal and diabetic states. Muscle activity in our animals was quite low. It might well be that there could be a difference in the glucose utilization of diabetics and normal animals after hepatectomy, if there were a high turnover of muscle energy. Such a difference has been suggested by Yater et al. (4), based on their own findings and those of Cruikshank (14) and of Knowlton and Starling (15). In our work we may be only comparing utilizations of "basal" tissues like the brain, heart and resting muscle.

One cannot determine the glucose utilization rate of the fed hepatectomized rabbit since here there would be an undetermined absorption of glucose from the intestine. When we compare the fed normal and the fed diabetic rabbit after evisceration, we find again that quantitatively the tissue utilization of glucose is the same. We have found an average of 198 mgm. per kilo per hour for the eviscerated fed diabetic rabbit (after eliminating rabbit 4 in table 1 on account of the low oxygen utilization). Bergman and Drury have found that the eviscerated fed rabbit used glucose at the rate of 206 mgm. per kilo per hour (5). They also showed that the increase in rate over fasted eviscerated rabbits (110 mgm. per kilo per hour) was not connected with any insulin mechanism. However, even with this increase the glucose utilization is not enough to take care of the entire needs of the body. We have obtained an average oxygen consumption of eviscerated rabbits of 385 cc. per kilo per hour; such an amount of oxygen could burn 470 mgm. of glucose. If then in the fed state, an animal can obtain all his energy from the burning of glucose he cannot do so entirely by mechanisms present in the eviscerated animal. There must be other mechanisms in the "non-eviscerated" or intact animal which play a rôle in the burning of glucose. It is possible that the pancreatic function is such a mechanism and that in the fed intact animal insulin increases the oxidation of sugar and that the difference noted above may be taken care of by a burning of glucose mediated by insulin.

SUMMARY

Evidence is presented for caution in drawing too hasty conclusions as to the glucose utilization rates of hepatectomized or eviscerated animals; particular care must be exercised in case the animal is already diabetic.

Our results indicate that under basal conditions the glucose utilization rate of diabetic tissues is the same as that of normal.

The amount of glucose needed to keep diabetic eviscerated animals in a steady state accounts for between a sixth and a third of the energy requirements of the animals. The same is the case for normals after evisceration.

REFERENCES

(1) Bergman, H. C. and D. R. Drury. This Journal **124:** 279, 1938.
(2) Macleod, J. J. R. and R. G. Pearce. This Journal **32:** 184, 1913.
(3) Mann, F. C. and T. B. Magath. Arch. Int. Med. **31:** 797, 1923.
(4) Yater, W. M., J. Markowitz and R. F. Cahoon. Arch. Int. Med. **51:** 800, 1933.
(5) Bergman, H. C. and D. R. Drury. Proc. Soc. Exper. Biol. and Med. **37:** 414, 1937.
(6) Mann, F. C. Medicine **6:** 447, 1927.
(7) Drury, D. R. and P. D. McMaster. Proc. Soc. Exper. Biol. and Med. **27:** 48, 1929.
(8) Drury, D. R. This Journal **111:** 289, 1935.
(9) Greeley, P. O. Proc. Soc. Exper. Biol. and Med. **37:** 309, 1937.
(10) Soskin, S. and R. Levine. This Journal **120:** 761, 1937.
(11) Young, F. G. J. Physiol. **87:** 11, 1936.
(12) Drury, D. R., H. C. Bergman and P. O. Greeley. This Journal **117:** 323, 1936.
(13) Drury, D. R. and P. D. McMaster. Proc. Soc. Exper. Biol. and Med. **27:** 48, 1929.
(14) Cruikshank, E. W. H. This Journal **90:** 322, 1929.
(15) Knowlton, E. P. and E. H. Starling. J. Physiol. **45:** 146, 1912.

INFLUENCE OF CARBON DIOXIDE ON THE EXCITABILITY OF THE VASOMOTOR CENTER IN HYPOGLYCEMIA[1]

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In an earlier investigation, Gellhorn in collaboration with Ingraham and Moldavsky (1938–39) and Kraines (1939) showed that in hypoglycemia, the blood pressure response to anoxia is markedly increased during the hypoglycemic state. Similarly it was found (Yesinick and Gellhorn, 1939) that anoxia as well as hypoglycemia increases the blood pressure response to a standardized raised intracranial pressure. Provided that the period of anoxia is not too long, anoxia may show a greater blood sugar raising effect during hypoglycemia than is observed at normal blood sugar levels (Gellhorn and Packer, 1939–40). All these effects could be understood on the basis that anoxia as well as hypoglycemia diminishes the rate of oxidations in the central nervous system. Therefore, the two factors combined would have a greater effect than each one separately. The question arose as to whether during the state of hypoglycemia the sympathetic centers are in a state of increased excitability which results in increased reactivity even to other factors which do not influence the metabolism of the brain as profoundly as anoxia. For this reason, experiments were performed on the interaction of carbon dioxide and hypoglycemia relative to the blood pressure in normal anesthetized dogs and in animals with the buffer nerves removed.

METHOD. The experiments were performed on 14 dogs anesthetized with 55 mgm./kgm. sodium amytal intraperitoneally. Artificial respiration was instituted, the femoral vein was cannulated and the blood pressure was recorded from the carotid artery. In the "denervated" dogs both vagi were cut and the carotid sinuses were removed bilaterally. The success of the complete denervation was tested by the blood pressure reaction to gas mixtures low in oxygen (Gellhorn and Lambert). The dogs inhaled CO_2-air mixtures from Douglas bags which varied in concentrations between 5 and 15 per cent for periods of 1 to 2 minutes. Insulin (Lilly)[2] was used and 3 units/kgm. were given intravenously and the same quantity intramuscularly. The blood sugar was determined by the

[1] Aided by a grant from the John and Mary Markle Foundation.

[2] Kindly supplied by Eli Lilly & Co.

Somogyi modification of the Shaffer-Hartman method. The protein was precipitated by zinc sulfate and barium hydroxide.

RESULTS. A typical result (fig. 1) shows that with falling blood sugar, the blood pressure rises considerably more in response to carbon dioxide than it does at normal blood sugar level. When low concentrations (about 5 per cent) of CO_2 are used, it is frequently seen that at normal blood sugar level, the blood pressure remains unchanged, but shows a considerable rise when the blood sugar has fallen to 40 mgm. per cent or below. When

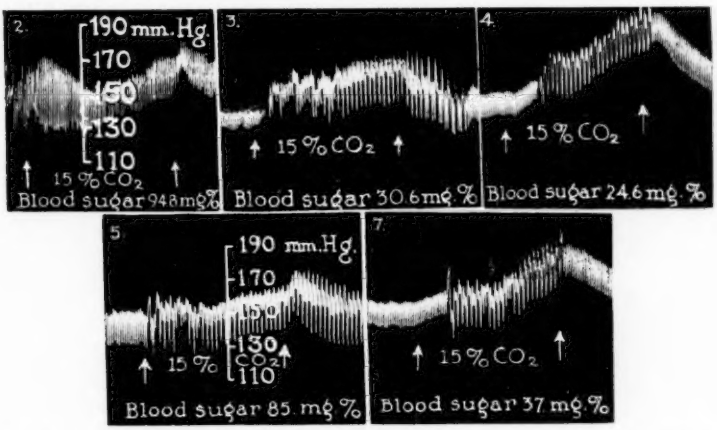

Fig. 1. 4/13/39 Female dog. 8.1 kgm. Sodium amytal 5.5 per cent intraperitoneally 1 cc./kilo. Artificial respiration. The gas mixture is inhaled for 2 minutes (between the two arrows).

2, 15 per cent CO_2. Blood sugar 94.8 mgm. per cent. Between *2* and *3*, three units of insulin per kilo are injected intravenously and three units per kilo intramuscularly.

3, 15 per cent CO_2. Blood sugar 30.6 mgm. per cent.

4, 15 per cent CO_2. Blood sugar 24.6 mgm. per cent. Between *4* and *5*, 50 cc. 10 per cent glucose were injected intravenously.

5, 15 per cent CO_2. Blood sugar 85 mgm. per cent.

7, 15 per cent CO_2. Blood sugar 37 mgm. per cent.

higher concentrations of CO_2 (15 per cent) are used, the blood pressure response at normal sugar level is somewhat variable in different animals. Sometimes it is found that the blood pressure shows a moderate fall. In other cases (fig. 1) the blood pressure rises after a slight initial fall. Figure 1 shows that when the blood sugar falls to 30.6 mgm. per cent, the blood pressure rises immediately in response to 15 per cent CO_2 and this rise is greatly increased when the blood sugar falls to a still lower level (24.6 mgm. per cent). The reaction is quite reversible as shown by the further

course of the experiment illustrated in figure 1. Not infrequently, the blood sugar will fall again due to the continued action of insulin after it has temporarily been restored to approximately normal levels by the injection of glucose. If that is the case, the increased rise of blood pressure in response to carbon dioxide may occur again.

A closer scrutiny of figure 1 shows that whereas in the control experiment at the beginning (no. 2) the inhalation of CO_2 alters the blood pressure level without revealing any changes in heart action, the heart action becomes irregular and marked vagal pulses appear as the blood sugar falls. This seems to indicate that hypoglycemia sensitizes not only the vasomotor but also the cardioinhibitory center to carbon dioxide[3] but it is interesting to note that in spite of marked vagal stimulation, the total effect on the blood pressure is that of increased sympathetic and not parasympathetic activity. Apparently hypoglycemia sensitizes the parasympathetic system to CO_2 to such a degree that even after restoration of the blood sugar to 85 mgm. per cent vagal pulses occur in response to carbon dioxide although the sympathetic excitability has been restored to a normal level.

In another series of experiments, the effect of carbon dioxide inhalation on the blood pressure during hypoglycemia was studied in animals in which the buffer nerves had been removed. Figure 2 shows a typical example. At 1, the dog inhaled 7.1 per cent O_2 for 2 minutes and showed a fall in blood pressure which is characteristic of the "denervated" animal (Gellhorn and Lambert, 1939). Then 5.4 and 15 per cent CO_2 were inhaled for 2 and 1 minutes respectively. In both cases, the blood pressure fell but with the low concentration of carbon dioxide, the alteration in blood pressure was very slight. When, after injection of insulin, the blood pressure had fallen to 21 mgm. per cent, the lower concentration of carbon dioxide caused a very marked rise in blood pressure but even with 15 per cent CO_2 a rise in blood pressure was obtained. On injection of glucose, the reactions were completely reversible.

The experiments show clearly that under conditions of hypoglycemia, the vasomotor center is sensitized to carbon dioxide in a similar way as has been found previously with regard to anoxia. In the studies on anoxia, this sensitizing action referred not to a direct interaction of anoxia and hypoglycemia on the vasomotor center, but rather to an increased response of the vasomotor center to the excitatory impulses originating in the chemoreceptors of the carotid sinus and the arch of the aorta. Since in the present experiments, the sensitization holds true for both the normal and the "denervated" animal, it may be said that the vasomotor center itself is sensitized to carbon dioxide by hypoglycemia. The occurrence of vagal impulses during the inhalation of CO_2 in the hypoglycemic animal

[3] Heymans, Bouckaert and Samaan (1933) report that carbon dioxide acts directly on the vagal center.

indicates that not only the sympathetic but also the vagal center is sensitized to CO_2 but it is characteristic that the normal preponderance of the sympathetic over the parasympathetic is not only maintained but greatly increased in hypoglycemia as the blood pressure response indicates (cf. Domm and Gellhorn). As to the fact that a depressor response to high concentrations of carbon dioxide is changed into a pressor response in hypoglycemia, the following explanation seems probable. The blood pressure effect of carbon dioxide is the resultant of its peripheral action on

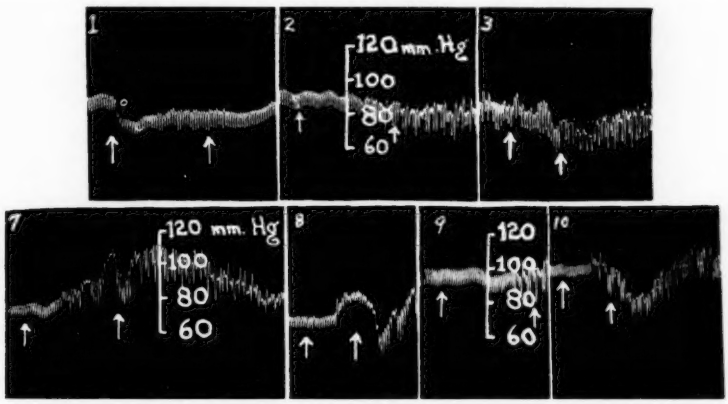

Fig. 2. 5/11/39 Male dog. 8 kgm. Sodium amytal 5.5 per cent intraperitoneally 1 cc/kgm. Both carotid sinuses denervated and vagi cut. Artificial respiration: 15 per cent CO_2 and 5.4 per cent CO_2 are inhaled for 1 and 2 minutes respectively.

1, 7.1 per cent O_2 for 2 minutes (between the arrows).

2, 5.4 per cent CO_2. Blood sugar 69 mgm. per cent during *2* and *3*.

3, 15 per cent CO_2. Blood sugar 69 mgm. per cent during *2* and *3*. Between *3* and *4*, three units of insulin per kilo are injected intravenously and three units per kilo intramuscularly.

7, 5.4 per cent CO_2. Blood sugar 21 mgm. per cent during *7* and *8*.

8, 15 per cent CO_2. Blood sugar 21 mgm. per cent during *7* and *8*. 50 cc. 10 per cent glucose intravenously between *8* and *9*.

9, 5.4 CO_2. Blood sugar 75.5 mgm. per cent during *9* and *10*.

10, 15 per cent CO_2. Blood sugar 75.5 mgm. per cent during *9* and *10*.

the blood vessels (vasodilatation) and its stimulation of the vasomotor center. If the former is strong enough, a fall in blood pressure may supervene. In such a case the greatly increased response of the vasomotor center to carbon dioxide which occurs in hypoglycemia may be sufficient to counteract the peripheral effects of CO_2 and result in a marked pressor response. The situation is similar to that observed by Lambert and Gellhorn in their studies on the influence of various depths of narcosis on the blood pressure effect of carbon dioxide.

SUMMARY AND CONCLUSIONS

Experiments are reported in which in anesthetized normal dogs with and without buffer nerves, the blood pressure response to various concentrations of carbon dioxide was determined at various blood sugar levels. It was found that the blood pressure rise to CO_2 increases with falling blood sugar. If, as is frequently the case when high concentrations (15 per cent) of carbon dioxide are used, the response to CO_2 at a normal blood sugar level consists in a fall in blood pressure, this reaction may be converted into a rise when the blood sugar falls below 40 mgm. per cent. The reaction is completely reversible on injection of glucose.

It is seen not infrequently that at a low blood sugar level, vagal pulses appear in response to carbon dioxide indicating that sympathetic and parasympathetic centers are sensitized to carbon dioxide by the hypoglycemic state. However, the physiological predominance of the sympathetic over the parasympathetic is not only maintained but increased in hypoglycemia.

Addendum. Van Harreveld and McRary (Proc. Soc. Exp. Biol. Med., **43:** 564, 1940) interpret our earlier work on the interaction of anoxia and hypoglycemia as due to a diminution of the buffer reflexes rather than to an increased excitability of medullary centers. The experiments described in this paper which show an increased response to CO_2 even in the "denervated" animal clearly disprove this interpretation. Moreover, our interpretation is supported by Yesinick and Gellhorn (this JOURNAL **128:** 185, 1939) who found that increased intracranial pressure produces a greater rise in blood pressure during hypoglycemia than at normal blood sugar and that this effect persists in the absence of the buffer nerves.

REFERENCES

COFFEE, A. AND E. GELLHORN. This Journal **126:** 467, 1939.

DOMM, S. AND E. GELLHORN. Arch. Neurol. and Psychiat. In press.

GELLHORN, E. Arch. Neurol. and Psychiat. **40:** 125, 1939.

GELLHORN, E. Livro de Homenagem aos Professores Alvaro e Miguel Ozorio de Almeida, Rio de Janeiro, Brasil, 1939.

GELLHORN, E. AND R. C. INGRAHAM. Proc. Soc. Exper. Biol. **40:** 315, 1939.

GELLHORN, E., R. C. INGRAHAM AND L. MOLDAVSKY. J. Neurophysiol. **1:** 301, 1939.

GELLHORN, E. AND E. LAMBERT. The vasomotor system in anoxia and asphyxia. (Illinois Monograph) 1939.

GELLHORN, E. AND A. PACKER. This Journal. In press.

HEYMANS, C., J. J. BOUCKAERT AND A. SAMAAN. C. R. Soc. Biol. **115:** 423, 1933.

KRAINES, S. H. AND E. GELLHORN. Am. J. Psychiat. **95:** 1067, 1939.

YESINICK, L. AND E. GELLHORN. This Journal **128:** 185, 1939.

STUDIES ON HYPOGLYCEMIC AND ANOXIC CONVULSIONS[1]

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In 1939 McQuarrie and Ziegler published investigations in which they studied the effect of prolonged anoxia on the sensitivity of dogs to insulin convulsions. They observed that whereas dogs not subjected to anoxia showed regularly insulin convulsions, these were absent when the animals were subjected to anoxia of varying degrees of severity for several hours. Furthermore, McQuarrie and Ziegler established the important fact that convulsions were absent although the blood sugar level in the dog subjected to anoxia and hypoglycemia was lower than in the dog treated with insulin alone. The experiments were conducted in a similar manner as those by Glickman and Gellhorn (1938) the main difference being that these authors used rats instead of dogs and produced anoxia, not by the inhalation of suitable oxygen-nitrogen mixtures, but by reducing the barometric pressure. Nevertheless the results were apparently in contradiction to those observed by McQuarrie and Ziegler since in rats subjected to insulin and lowered barometric pressure convulsions occurred earlier and showed a much greater fatality rate than was observed in animals subjected to insulin alone. In order to explain this apparent discrepancy, experiments were carried out in which the interaction of anoxia and hypoglycemia involving convulsive insulin concentrations was studied in two different species. For one, the rabbit was chosen because numerous experiments proved that the unanesthetized rabbit lends itself particularly well to repeated studies on insulin hypoglycemia on account of the constancy of the hypoglycemic reaction. The other experiments were carried out in rats in a manner similar to that used in the work of Glickman and Gellhorn with the modification that now oxygen-nitrogen mixtures were employed instead of the low pressure chamber. In other words, conditions in the experiments performed on rats and rabbits were practically identical. Therefore, this procedure should allow one to decide whether the discrepancy in the results of Glickman and Gellhorn and those of McQuarrie and Ziegler is due to species differences or to other factors.

[1] Aided by a grant from the John and Mary R. Markle Foundation.

METHODS. All animals were starved for eighteen hours prior to experiment. Insulin (Eli Lilly & Co.)[2] was injected subcutaneously in rabbits (2.5 units/kgm.) and intraperitoneally (10 units/kgm.) in rats. Oxygen-nitrogen mixtures containing 7 per cent oxygen were made by the use of calibrated flow meters. They were administered to the rabbits by means of a metal tube which served as a head holder and was provided on one side with a perforated rubber dam sealing the mouth airtight. The gas mixtures were inhaled from Douglas bags through two valves. The rats were placed in airtight glass bottles, through which the gas

TABLE 1

MINUTES AFTER INJECTION OF INSULIN				OCCURRENCE OF CONVULSIONS
0	60	90	120	
(A) The effect of 2.5 units of insulin per kgm. on the blood sugar of rabbits				
82.7	30.6	27.8		At 106 minutes
81.1	32.2	30.6		At 103 minutes
78.5	38.7	30.6		At 102 minutes
91.3	32.2	27.8		At 91 minutes
93.5	24.1	22.2		At 115 minutes
72.0	28.5			At 83 minutes
(B) The effect of 2.5 units of insulin per kgm. on the blood sugar of rabbits subjected to 7 per cent oxygen*				
81.1	29.7	25.0	23.2	None
78.5	27.8	24.1	26.0	None
88.2	33.3	24.1	24.1	None
80.6	25.0	27.8	29.7	None
76.3	24.1	25.0	22.2	None
96.8	39.7	20.5	15.3	None

* The inhalation of 7 per cent O_2 was begun immediately after the injection of insulin and was continued for 2 hours.

mixture was drawn by suction at a speed several times greater than that corresponding to their minute volume of respiration. The gas mixtures were administered for two hours immediately after the injection of insulin. No anesthetic was used.

RESULTS. Our experiments show, first, that the administration of anoxia for two hours prevented the occurrence of convulsions in rabbits subjected to convulsive doses of insulin. Secondly, they show that in spite of this fact, the blood sugar values observed were even slightly lower in the insulin-anoxia experiments than in the insulin controls. The minimum blood sugar value observed in six anoxia-insulin experiments was

[2] Kindly supplied by Eli Lilly & Co.

23.4 mgm. per cent whereas it was 27.9 mgm. per cent in six control experiments carried out without anoxia.[3] This confirms McQuarrie and Ziegler's work and extends it to the rabbit.

McQuarrie and Ziegler had observed also that it is possible in dogs to prevent insulin convulsions by the inhalation of 15 per cent CO_2 in air, whereas convulsions regularly occur when the animal inhaled 15 per cent CO_2 in oxygen. This observation implies that when the animal inhaled 15 per cent CO_2 for a long time, some condition of anoxia evolves. Table 2 shows ten experiments in which rabbits were subjected to two hours of 15 per cent CO_2 after 2.5 U insulin per kilo were injected. In eight out of ten experiments the respiratory volumes were also determined at vari-

TABLE 2

The effect of 2.5 units insulin per kgm. on the blood sugar of rabbits which inhaled 15 per cent CO_2 for 2 hours after the injection

BLOOD SUGAR IN MGM. %, MINUTES AFTER INJECTION				RESPIRATORY VOLUME (L/MIN.) DURING INTERVAL				CONVULSIONS
0	60	90	120	0	0–30	60–90	90–120	
84.9	50.5	24.1	22.2					None
78.5	47.3	40.8	38.7					None
72.0	41.9	32.2	22.2	1.20	0.91	0.16		None
92.5	35.4	20.4	14.8	2.34	3.63	3.36	1.31	None
93.5	28.5	22.2		2.40	1.48	1.41	0.68	None
82.7	38.7	38.7		1.70	3.81	2.00	1.50	At 112 min.
91.3	28.5	28.5	31.3	1.91	1.35	0.49	0.28	None
136.5*	60.2	41.9	35.4	1.26	0.24	0.41	0.50	None
79.5	27.8	22.2	24.1	2.37	3.20	3.17	3.24	None
83.8	31.3	24.1	25.0	0.99	3.36	2.86	2.19	None

* (Struggle!)

ous intervals. It is seen that only one out of ten animals showed convulsions. Furthermore, in most of them, although not in all, the blood sugar reached convulsive levels. (Compare these values with the blood sugar values obtained in the 2.5 U/kgm. insulin control experiments shown in table 1.) In four out of eight experiments, it is seen that the respiratory volume greatly decreases during the course of the experiment, indicating that in these cases a very marked anoxia is produced. This, however, does not apply to the other four experiments in which the respiratory volume is either not reduced or is even increased. Since in three out of four experiments, convulsions are prevented in spite of adequate respiration, it seems to follow that CO_2 has some independent effect in preventing insulin convulsions. This has been confirmed in regard to other con-

[3] Compare also Gellhorn and Packer (1940).

vulsive drugs in cats in experiments which will be reported elsewhere (Yesinick and Gellhorn).

Experiments in rats showed that although the rats subjected to 7 per cent oxygen for two hours did not show any untoward effects, it was found that when they were subjected to 7 per cent oxygen plus insulin convulsions started much earlier and occurred more frequently than when insulin alone was administered. Table 3 shows that eight out of twelve rats showed hypoglycemic convulsions when injected with 10 units of insulin/kgm. and that ten out of twelve rats convulsed when injected with the same amount of insulin and subjected to 7 per cent oxygen at the same time. The latent period varied between 141 and 228 minutes in the first and 22 and 107 minutes in the latter group. The experiments confirm the work of Glickman and Gellhorn but are more striking than the results of these authors since in our new experiments no overlap exists in the latent periods of convulsions in the two groups. There is not only a quantitative but also a qualitative confirmation of the earlier work of Glickman and Gellhorn. It was mentioned in our earlier work that the type of convulsions which rats showed after insulin plus anoxia, the latter induced by the lowering of the barometric pressure, was quite different from that observed in rats subjected to insulin alone. It resembled more anoxic convulsions which may be seen either by employing oxygen-nitrogen mixtures very low in oxygen or by reducing markedly the barometric pressure. Glickman and Gellhorn came to the conclusion that "It appears as if the insulin injected animals were suffering from a relative anoxia which is greatly aggravated when the animals are exposed to a reduced oxygen pressure, although the latter is entirely harmless to non-insulinized control animals." These observations are fully confirmed in the present set of experiments. We have other indications of the essential anoxic nature of the convulsions in the animals treated with insulin and 7 per cent oxygen. Rats injected with insulin alone show convulsions that last for several minutes consisting of vigorous clonic-tonic discharges. These were almost immediately relieved by intraperitoneal injection of glucose. In sharp contradistinction to this phenomenon, it is found that rats subjected to anoxia plus insulin show very brief clonic convulsions lasting only a few seconds. In these cases the injection of glucose is of no benefit. The animals frequently die even before glucose can be injected. The anoxic nature of the convulsions observed in rats is substantiated by the third group of experiments reported in table 3. Ten rats were again subjected to insulin and 7 per cent oxygen and early convulsions of the anoxic type occurred in all animals. As soon as they appeared, 100 per cent oxygen was administered and continued for about twenty minutes. Seven out of ten rats recovered whereas none of the animals recuperated when injected with glucose (group II of table 3).

TABLE 3

I. Effect of 10 units insulin/kgm. (intraperitoneally) on normal rats

NUMBER	LATENT PERIOD OF HYPOGLYCEMIC CONVULSIONS	REMARKS
	minutes	
1	223	Recover on injection of glucose
2	228	
3	∞	
4	∞	
5	152	Recover on injection of glucose
6	217	
7	188	
8	∞	
9	159	Recover on injection of glucose
10	141	
11	153	
12	∞	

II. Effect of 10 units insulin/kgm. (intraperitoneally) on rats subjected for 2 hours to inhalation of 7 per cent oxygen

NUMBER	LATENT PERIOD OF CONVULSIONS (ANOXIC TYPE)	REMARKS
	minutes	
1	28	All died in spite of injection of glucose
2	51	
3	107	
4	∞	
5	37	All died in spite of injection of glucose
6	48	
7	63	
8	∞	
9	27	All died in spite of injection of glucose
10	22	
11	23	
12	61	

III. Effect of 10 units insulin/kgm. (intraperitoneally) on rats subjected for 2 hours to inhalation of 7 per cent oxygen

NUMBER	LATENT PERIOD OF CONVULSIONS (ANOXIC TYPE)	TREATED IMMEDIATELY WITH OXYGEN	SHOW HYPOGLYCEMIC CONVULSIONS AFTER
	minutes		*minutes*
1	47	Recover	179*
2	46	Died	
3	31	Recover	
4	58	Recover	
5	46	Recover	
6	26	Recover	190*
7	30	Recover	143*
8	51	Died	
9	60	Died	
10	94	Recover	

* All recover on injection of glucose.

Moreover, it was found that some of the rats thus saved from the otherwise fatal effect of anoxia during insulin hypoglycemia, showed two to three hours later hypoglycemic convulsions which could be promptly alleviated by injection of glucose.

We therefore come to the conclusion that the rabbits in our experiments behaved similarly to the dogs in the experiments of McQuarrie and Ziegler. In these animals, insulin convulsions are prevented by anoxia. In the rats, however, no matter whether a low barometric pressure or the inhalation of oxygen-nitrogen mixtures is used for inducing anoxia, the animals subjected to insulin plus anoxia die under convulsions, whereas convulsions occur only later or not at all in the animals treated with insulin alone. But it seems to be unfair to conclude from these experiments that insulin convulsions are prevented by anoxia in rabbits and dogs but are precipitated by anoxia in rats. In confirmation of the earlier work of Glickman and Gellhorn, we found in our new experiments on rats that the convulsions observed in hypoglycemic and anoxic animals are definitely of an anoxic and not of a hypoglycemic nature. We may, therefore, say that in all animals thus far studied with regard to the interaction of anoxia and hypoglycemia, anoxia prevents insulin convulsions, but in the rats, anoxic convulsions occur which are not seen either in the dog or in the rabbit subjected to similar procedures. If we take into consideration the fact that both hypoglycemia and anoxia diminish the rate of oxidations in the brain, the difference between rats on one side and rabbits and dogs on the other side seems to be not a difference in sensitivity to hypoglycemic convulsions but a difference in sensitivity to oxygen deficiency. In the case of rats, anoxia when superimposed on hypoglycemia, creates such a diminution of oxidative processes in the brain that these animals show anoxic convulsions. Rabbits in general do not show these symptoms, although in a few cases with denervated adrenals we saw a similar behavior and then the injection of glucose was of no avail. The conclusion which we arrive at that a similar diminution in the oxidation rate of the brain may produce more marked anoxic symptoms in rats than in rabbits and dogs, is supported by the experience of Haldane that small animals such as mice and birds are more sensitive to carbon monoxide than are larger animals, including man. It is this specific sensitivity to anoxia, characteristic of small animals with a high metabolic rate, which accounts for the differences observed in the two groups of experiments. The conclusion seems imperative that insulin convulsions require a higher degree of oxygenation of the brain centers than is present when the animals are subjected to anoxia during the hypoglycemic state. The occurrence of anoxic convulsions in rats subjected to insulin plus 7 per cent oxygen furnishes a further illustration of the synergistic action of anoxia and hypoglycemia on the central nervous system (cf. Gellhorn and collaborators, 1938–1940).

SUMMARY

1. Rabbits injected with 2.5 units of insulin subcutaneously and subjected to 7 per cent oxygen for two hours do not show insulin convulsions in spite of convulsive blood sugar levels. This confirms the work of McQuarrie and Ziegler. If, instead of low oxygen, 15 per cent CO_2 is inhaled, the results are similar to the experiments with anoxia. In 50 per cent of the rabbits, marked signs of respiratory failure are observed indicating that under these conditions, a marked diminution in the oxidative processes takes place also.

2. In experiments performed on rats which were given 10 units of insulin/kgm. intraperitoneally and subjected to 7 per cent oxygen for two hours, the animals show convulsions much earlier than the control animals subjected to insulin alone, but the nature of these convulsions is anoxic and clinically different from insulin convulsions. This is confirmed by the fact that whereas injection of glucose immediately restores the animals suffering from insulin convulsions, it has no beneficial effect on animals showing anoxic convulsions. Moreover, such convulsions can be alleviated by administration of pure oxygen. The difference in the behavior of rats and rabbits is explained on the basis of the greater oxygen sensitivity of small animals showing a high metabolic rate. It is concluded that in all species thus far studied, anoxia prevents insulin convulsions.

REFERENCES

COFFEE, A. AND E. GELLHORN. This Journal **126:** 467, 1939.

GELLHORN, E. Arch. Neurol. Psychiat. Chicago **40:** 125, 1938.
In Livro De Homenagem aos Professores Alvaro e Miguel Ozorio de Almeida Rio de Janeiro, Brasil, 1939.

GELLHORN, E., R. C. INGRAHAM AND L. MOLDAVSKY. J. Neurophysiol. **1:** 301, 1938.

GELLHORN, E. AND A. C. PACKER. Proc. Soc. exper. Biol. New York **41:** 345, 1939.
This Journal, **129:** 610, 1940.

GLICKMAN, N. AND E. GELLHORN. This Journal **121:** 358, 1938.

HALDANE, J. S. Respiration. London, 1927.

INGRAHAM, R. C. AND E. GELLHORN. Proc. Soc. exper. Biol. New York **40:** 315, 1939.

KRAINES, S. H. AND E. GELLHORN. Am. J. Psychiat. **95:** 1067, 1939.

MCQUARRIE, I. AND M. ZIEGLER. Proc. Soc. exper. Biol. New York **39:** 525, 1939.

YESINICK, L. AND E. GELLHORN. This Journal **128:** 185, 1939.

ACTIONS OF ADRENALINE AND ACETYLCHOLINE ON THE DENERVATED IRIS OF THE CAT AND MONKEY

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It has been shown repeatedly that when structures supplied by sympathetic nerves are deprived of their post-ganglionic supply, they become sensitive to circulating epinephrine. Likewise, when parasympathetically innervated muscle is deprived of its post-ganglionic supply, it contracts readily when in contact with acetylcholine. Shen and Cannon (1936) found that after excision of the ciliary ganglion in the cat, the paralyzed circular fibers of the iris constricted the pupil on the instillation of 1.0 per cent acetylcholine into the conjunctival sac. This miosis was augmented by previous application of eserine. In this communication comparative studies are reported of the effects of adrenaline and acetylcholine on the sympathetically and parasympathetically denervated irises of the cat and monkey.[3]

METHOD. In the following experiments, cats and monkeys (Macaca mulatta) were used. In 60 cats and 20 monkeys, the superior cervical ganglion was removed, and in 30 cats and 7 monkeys the short and long ciliary nerves were sectioned. The latter procedure was a modification of the operation devised by Anderson (1905), and used by Shen and Cannon (1936). The method of exposure of the ciliary nerves in the monkey was similar to that used in the cat except that in the former it was necessary to trephine the lateral wall of the bony orbit.

The superior cervical ganglion was removed in the usual manner. The changes in pupillary diameter were recorded in most instances by measuring the horizontal diameter with a millimeter scale, and in some photographically.

[1] Aided by grants of the Josiah Macy Jr. Foundation and the Dazian Foundation for Medical Research.

[2] Dr. I. Abrahamson Fellow in Neuropsychiatry.

[3] For purposes of brevity, the iris deprived of its post-ganglionic sympathetic nerve supply will be designated as the *s.d.*, i.e., sympathetically denervated iris, while the iris of the eye in which the post-ganglionic ciliary and optic nerves were sectioned will be referred to as the *c.d.*, i.e., completely denervated iris. In the completely denervated iris, both the sympathetic and parasympathetic nerve supplies were abolished.

In order to visualize the pupil better, the nictitating membrane was removed in a number of cats. In chronic experiments, the eye was bandaged with 1:5000 bichloride of mercury ointment, so as to avoid ulcerations of the cornea.

The drugs, i.e., adrenaline hydrochloride (Parke Davis) and acetylcholine hydrochloride (Merck) were used with the animal in most instances unanesthetized. The 1.0 per cent adrenaline solution used for local application to the eye was prepared from crystalline epinephrine bitartrate (Winthrop). All dilutions were made with saline to volume of 1.0 cc. The dosages recorded in the subsequent experimental data are per kilogram of body weight, unless otherwise noted.

RESULTS. I. *Adrenaline* (a) *cat.* The normal iris and that deprived of its sympathetic innervation by preganglionic section dilated with intravenous injections of adrenaline in amounts not less than 5 gamma, while the iris in which the post-ganglionic sympathetic fibers were cut dilated with much smaller doses. Intravenous injection of adrenaline, 5 gamma or more, excited the radial fibers of the iris of the cat even immediately after their complete denervation. Following this operation, the sensitivity of the denervated pupillodilator muscle to epinephrine increased with time. The minimal dose of epinephrine necessary to dilate the *s.d.* iris was 0.08 gamma. The sensitivity of the radial fibers of the *c.d.* iris was even greater, 0.02 gamma produced a minimal dilatation. When injected into the ipsilateral common carotid artery, the quantity of epinephrine required to dilate the *c.d.* iris was 0.0005 gamma.

(b) *Monkey.* In the monkey, the sensitivity of the *s.d.* iris to intravenous injections of adrenaline was not as marked as in the cat. In only two macaques were 4.0 gamma of adrenaline effective in producing mydriasis of the *s.d.* iris. In all other instances from 50 to 200 gamma were necessary. The mydriasis was never conspicuous or maximal, the pupil increasing only by 1.0 to 2.5 mm. over the original diameter. In three monkeys, the pupil became irregular and there was hemorrhage into the anterior chamber of the eye. In monkeys with preganglionic section at the stellate or at the middle cervical ganglion, large doses of intravenously injected adrenaline sometimes enlarged the ipsilateral pupil. The normally innervated iris did not dilate with intravenous injections of adrenaline.[4]

Unlike the cat, complete denervation of the monkey's iris did not

[4] These pupillary dilator reactions noted in the cat and monkey, following the administration of adrenaline, are in accord with the observations of the mydriasis obtained when each is frightened. Frightening the cat produces a marked dilatation of the *s.d.* and *c.d.* irises presumably due to a discharge of adrenaline. In the monkey there is no change in pupillary diameter with fright and struggle unless cocaine is given locally or parenterally (Fulton, 1940).

enhance the sensitivity to epinephrine even when large doses ranging from 200 to 1000 gamma were employed. The mydriasis was slight and frequently did not occur. Instead a progressive constriction of the pupil ensued from 45 to 90 seconds after the adrenaline was injected (fig. 1A). This delayed miosis of the *c.d.* iris caused by adrenaline could be enhanced by eserine, given 15 minutes previously either in the conjunctival sac or intramuscularly, and could be diminished or abolished by parenteral atropine. Eserine thus given had no visible effect on the

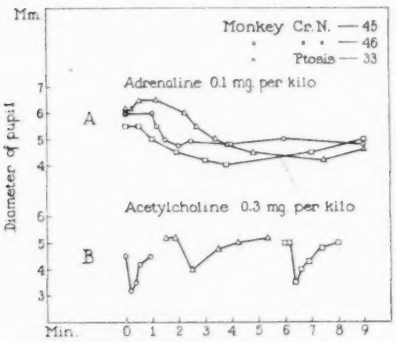
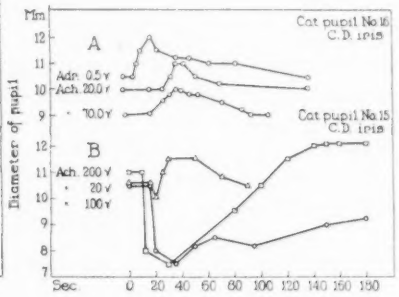


Fig. 1



F g. 2

Fig. 1. Three unanesthetized monkeys each with *c.d.* iris. At *A*, injected with adrenaline into saphenous vein. At *B*, injected with acetylcholine by vein. Note the long interval between injection of epinephrine, first point indicated, and appearance of miosis following the drug injection.

Fig. 2. Unanesthetized cat with *c.d.* iris. Weight 2.8 kgm. Effect of intravenous injections of acetylcholine on pupillary diameter in amounts as indicated. Pupillary constriction could be elicited immediately after the denervation.

A. Comparative actions of adrenaline and acetylcholine. Note that the adrenaline mydriasis appeared within the circulation time of 6 seconds, while acetylcholine mydriasis was delayed and appeared twenty or more seconds after the drug was injected at first point.

B. Variable constrictor and dilator effects of intravenous acetylcholine. Curve with squares represents that produced by intravenous injection of 200 gamma, with triangles 20 gamma and with circles 100 gamma of acetylcholine.

pupil. The miosis due to adrenaline could not be elicited in every monkey tested, nor on every occasion. Furthermore, the phenomenon could be demonstrated only during the period in which the denervated pupilloconstrictor muscle was hypersensitive and readily contracted with acetylcholine.

II. *Acetylcholine* (a) *cat.* In the cat, intravenous injections of acetylcholine up to 50 gamma had little effect on the normal iris. In doses greater than 6 to 10 gamma, dilatation of the *s.d.* iris occurred 14 to 30

seconds following the administration of the acetylcholine. This interval was considerably greater than the circulation time which is usually six seconds (fig. 2A). Eserine increased and atropine abolished or hindered the delayed mydriatic effect of acetylcholine. In most instances, bilateral adrenalectomy abolished the dilatation in the *s.d.* iris and diminished it in the normal iris. The *s.d.* and normal nictitating membranes retracted with acetylcholine even after bilateral adrenalectomy.

In the *c.d.* iris of the cat, acetylcholine administered intravenously had a dual action, mydriatic and miotic (fig. 2B). The responses varied, but the dilator effect was the more frequent. Even when miosis did occur, it preceded or followed a mydriasis. At times no change in the pupil was noted. The constrictor effect of acetylcholine on the *c.d.* iris did not seem to depend on the quantity injected, for in the same cat 10 gamma were equally effective and ineffective. Intracarotid injections of acetylcholine also yielded variable results; in one instance 4 gamma produced miosis, on other occasions there were no changes, even with as large a dose as 1 mgm. The constriction produced by acetylcholine appeared from 10 to 60 seconds after its intravenous injection into the uneserinized cat and persisted for many minutes, long after the acetylcholine must have disappeared from the circulation by the hydrolytic action of blood cholinesterase. Atropine, 1 mgm. intramuscularly, abolished the miotic and diminished the mydriatic effect of parenteral acetylcholine. With large doses of intravenously injected acetylcholine, 100 gamma or more, slight constriction of the *c.d.* iris could be elicited as early as 5 minutes after the denervation. At this early stage the usual secondary mydriasis was not observed.

(b) *Monkey.* In the monkey, intravenous injections of acetylcholine produced constriction of the *c.d.* iris (fig. 1B). The constriction was augmented and prolonged by eserine injected parenterally or applied locally to the eye. This acetylcholine miosis was also an inconstant phenomenon but there were no apparent secondary effects on the pupil, such as the delayed mydriasis observed in the cat, even when large doses of acetylcholine, 1 to 4 mgm., were administered with or without previous eserinization.

Discussion. After sectioning all the ciliary sympathetic and parasympathetic nerves to the eye, both adrenergic and cholinergic sensitization phenomena may be demonstrated in the denervated iris; the pupillodilator muscle reacts with adrenaline while the pupilloconstrictor muscle reacts with acetylcholine. The actions of adrenaline and acetylcholine on the denervated iris of the cat differ from those of the monkey. The pupillary effects obtained with intravenous injections of these drugs may be determined by general and local reactions.

A. *General (reflex) reactions.* The injection of an excess amount of one

of the autonomic substances causes a reflex discharge of the other in the body. Feldberg and Minz (1931) demonstrated that acetylcholine injected into the circulation caused a secretion of epinephrine from the adrenal medulla of cats, dogs, and rabbits. Conversely, Krayer and Verney (1934) showed that when adrenaline was injected intravenously, there occurred a reflex discharge of acetylcholine into the coronary vein of the dog and cat.

It is probable that the mydriasis of the denervated iris caused by acetylcholine in the cat is in part due to a reflex discharge of epinephrine from the adrenal medulla. This is supported by the observations that bilateral adrenalectomy abolishes and in some instances diminishes the mydriasis caused by acetylcholine. The fact that bilateral adrenalectomy does not eliminate this mydriasis in every cat tested suggests that acetylcholine may release epinephrine from obscure accessory chromaffin bodies or stimulates the secretion of an adrenaline-like substance, such as sympathin, at the sympathetic nerve endings (Bacq, 1936; Cannon and Lissàk, 1939). Using the *s.d.* nictitating membrane of the cat as an indicator, Bacq (1936) found that only after removal of the adrenals and complete sympathectomy does acetylcholine fail to retract the nictitating membrane. The mechanism of reflex discharge of epinephrine by acetylcholine may also exist in the monkey as it does in other species but the quantity so liberated may be insufficient to dilate the *s.d.* iris, which in this animal is found to have a low sensitivity to injected adrenaline. It is possible that if a different indicator were used, such as the denervated heart or intestine (Youmans et al., 1940), the reflexly liberated adrenaline or sympathin could be detected. The evidence and methods used to study the release of adrenaline by intravenously injected acetylcholine has been reviewed by Hermann et al., 1937.

Just as acetylcholine can stimulate the secretion of adrenaline, so may adrenaline cause the liberation of acetylcholine (Krayer and Verney, 1934). The constriction of the completely denervated iris of the monkey by intravenous injections of adrenaline may be due to a reflexly released parasympathomimetic substance. This is particularly likely since the miosis caused by adrenaline is augmented by eserine and occurs during the period the denervated pupilloconstrictor muscle is hypersensitive to acetylcholine. Additional presumptive evidence that the delayed pupillary constriction is due to a reflexly discharged chemical agent is found in experiments with denervated facial muscles of monkeys. These denervated muscles, which are extremely sensitive to acetylcholine, contract from 40 to 150 seconds after the intravenous administration of large amounts of adrenaline (Bender, 1939). The contraction is augmented by eserine, and occurs only while the denervated facial muscles are sensitive to acetylcholine. Still another example in which denervated striated muscle reacts indirectly with adrenaline are the experiments of Mahoney

and Sheehan (1936), who found that 5 days after intracranial section of the oculomotor nerve the denervated levator palpebrarum contracted *several minutes* after the intramuscular injection of 2 mgm. of epinephrine. Since the denervated ocular muscles are highly sensitive and contract readily with acetylcholine (Bender and Fulton, 1939) it is implied that the delayed contraction produced by adrenaline is due to reflex liberation of a cholinergic substance. The origin of this hypothetical, reflexly liberated cholinergic substance in the body is obscure and there is a question whether the parasympathomimetic effects are due to acetylcholine per se. Feldberg and Schriever (1936) found that intravenously injected adrenaline produced secretion of acetylcholine into the cerebrospinal fluid of eserinized dogs. According to these authors, the acetylcholine thus derived was not from the secretions of the vagal nerve endings.

The absence of the cholinergic effect following parenteral injections of adrenaline in the cat may be more apparent than real. It is possible that the dilator fibers of the iris react more powerfully than the constrictor set. Since adrenaline excites the dilator fibers, the stimulation of the constrictor set by reflexly liberated acetylcholine may not be sufficient to overcome the adrenaline mydriasis. Thus although acetylcholine may be secreted after intravenous injection of large doses of adrenaline, its presence in the cat could not be detected by the sensitized body indicators described in this report.

B. *Local (direct) reactions.* The radial and circular fibers of the iris each react with both adrenaline and acetylcholine. Cannon and Rosenblueth (1935) reported that hepatic sympathin acts on both the radial and circular fibers of the iris of the cat. Also, Rosenblueth (1932), Morrison and Acheson (1938), and Rosenblueth and Cannon (1939) found that the nictitating membrane of the cat contracts with adrenaline and acetylcholine, as we did. Similarly, it is conceivable that the constrictor and dilator sets of denervated fibers of the iris in the cat each react with acetylcholine. Since the two sets of fibers are antagonistically arranged, the final effect is the resultant of the two opposing actions. In the cat the usual response to intravenous injections of acetylcholine or adrenaline is dilator and this suggests that the dilator fibers are the more powerful of the two groups.

The same principle of a drug acting simultaneously on antagonistic sets of fibers of the iris may account for the adrenaline constriction of the *c.d.* iris of the monkey. In the monkey, however, in contrast to the cat, the pupilloconstrictor muscle may be stronger than the dilator fibers and, therefore, would respond with acetylcholine as well as with adrenaline. Although this hypothesis could explain the miosis of the *c.d.* iris it would hardly account for the phenomenon of contraction of the denervated facial striated muscles elicited by adrenaline in monkeys (Bender, 1939).

Of the two explanations offered for the pupillary responses obtained

with the autonomic drugs the experimental evidence seems to favor the theory of general hormonal reaction, namely, an excess of acetylcholine causes secretion of adrenergic substance while excess of adrenaline causes liberation of a cholinergic substance in the body. Such reflex reactions neutralize the effects of the drugs introduced into the body and thus conform with the widely established principle of homeostasis (Cannon, 1939).

The foregoing data reveal that the cat responds to autonomic drugs chiefly with the dilator, while the monkey with the constrictor mechanism of the denervated iris. Since in this case the iris is free from nervous control the changes in pupillary diameter must be due to an excitation of the iris fibers by a chemical agent either introduced or released within the body. The chemical agents liberated within the organism are presumably adrenaline and acetylcholine. These findings are similar to those noted in conditions in which the autonomic system is hyperactivated such as in fright and struggle (Bender, 1938), and in insulin-hypoglycemia (Bender and Siegal, 1940). All observations considered together suggest that both adrenergic and cholinergic like substances are elaborated in each of the species and that the cat displays predominantly the sympathomimetic, whereas the monkey shows more of the parasympathomimetic reactions to the same stimuli.

SUMMARY

1. The denervated iris of the cat and monkey differs in the reactions to the autonomic drugs. In general the cat responds with its dilator while the monkey reacts with its constrictor-fibers of the iris.

2. After sectioning of all the ciliary and optic nerves, the iris is rigid to all forms of neural stimuli but responds to chemical agents. Intravenous injections of adrenaline produce a dilatation of the *c.d.* iris in the cat but a delayed miosis in the monkey. Intravenous injections of acetylcholine produce an inconstant constriction and a more frequent secondary dilatation in the cat while in the monkey there is only constriction.

3. The pupillary dilatation obtained after injections of acetylcholine in the cat is usually abolished by bilateral adrenalectomy. The pupillary constriction which is noted on intravenous injections of adrenaline in the monkey is potentiated by eserinization and occurs only while the constrictor fibers of the iris are sensitive to acetylcholine. These facts suggest that excess of acetylcholine stimulates the secretion of adrenaline and excess of adrenaline stimulates the liberation of a cholinergic-like substance in the body.

REFERENCES

ANDERSON, H. K. J. Physiol. **33:** 414, 1905.
BACQ, Z. M. C. R. Soc. Biol. **123:** 1021, 1936.

BENDER, M. B. This Journal **121:** 609, 1938.
This Journal **127:** 15, 1939.
BENDER, M. B. AND J. F. FULTON: J. Neur. Psych. **2:** 285, 1939.
BENDER, M. B. AND S. SIEGAL. This Journal **128:** 324, 1940.
CANNON, W. B. J. Mt. Sinai Hosp. **5:** 587, 1939.
CANNON, W. B. AND K. LISŠÀK. This Journal **125:** 765, 1939.
CANNON, W. B. AND A. ROSENBLUETH. This Journal **113:** 251, 1935.
FELDBERG, W. AND B. MINZ. Arch. f. exper. Path. u. Pharmakol. **163:** 66, 1931.
FELDBERG, W. AND H. SCHRIEVER. J. Physiol. **86:** 277, 1936.
FULTON, J. F. Ann. Rev. Physiol. **2:** 258, 1940.
HERMANN, H., F. JOURDAN, G. MORIN AND J. VIAL. Arch. Int. Pharmacodyn. **57:** 403, 1937.
KRAYER, O. AND E. B. VERNEY. Klin. Wchnschr. **13:** 1250, 1934.
MAHONEY, W. AND D. SHEEHAN. Arch. Neur. Psych. **35:** 99, 1936.
MORRISON, R. S. AND H. ACHESON. This Journal **121:** 149, 1938.
ROSENBLUETH, A. This Journal **100:** 433, 1932.
ROSENBLUETH, A. AND W. B. CANNON. This Journal **125:** 276, 1939.
SHEN, S. C. AND W. B. CANNON. Chinese J. Physiol. **10:** 359, 1936.
YOUMANS, W. B., K. W. AUMANN, H. F. HANEY AND F. WYNIA. This Journal **128:** 467, 1940.

THE MECHANISM OF THE EXCRETION OF VITAMIN C BY THE DOG KIDNEY[1]

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The mechanism of the excretion of vitamin C in the human has been reported in a previous study (1, 2). It was found that in man ascorbic acid was excreted through filtration and active tubular reabsorption. The reabsorptive mechanism appeared to be limited by a maximal rate so that when the vitamin was presented to the tubules by the glomerular filtrate at a rate exceeding this maximum the excess was excreted in the urine. In a series of normals studied by us, and by Smith and his coworkers (3) it was found that the human kidney was capable of reabsorbing 1.2 to 2.1 mgm. of vitamin C per 100 cc. of glomerular filtrate with an average of 1.7 mgm. per 100 cc. of glomerular filtrate or 2.0 mgm./min. At plasma levels below saturation (2) the vitamin was almost completely reabsorbed. This reabsorption, however, was never entirely complete and some ascorbic acid appeared in the urine regardless of the plasma concentration. These data in the human appeared to uphold the hypothesis which treats tubular reabsorption as a phenomenon following the law of mass action (4). It was also shown that although in chemical structure vitamin C is related to the carbohydrates, it is not reabsorbed by the same mechanism as glucose.

Because the dog is capable of synthesizing its own vitamin C, the mechanism by which this animal excretes the vitamin was studied. In this investigation simultaneous creatinine and vitamin C clearances were done as this provides a method for studying the mechanism of the excretion of ascorbic acid in the dog.

PROCEDURE. Observations were made on four normal well-trained dogs kept on identical diets consisting of crackermeal 100 grams, skimmed milk 30 grams, yeast 10 grams, bone ash 4 grams, salt mixture 3 grams, meat powder 35 grams, cod liver oil 22 cc. The dogs were hydrated the day prior to the test and the morning of the experiment. The dogs were unanesthetized and loosely restrained upon a comfortable animal board.

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Constant creatinine and varying ascorbic acid concentrations in the plasma were obtained by means of constant intravenous infusions. Urine was collected by an indwelling catheter and at the end of each period the bladder was emptied as completely as possible and washed out with saline. Bloods were obtained at frequent intervals.

Potassium oxalate was used in minimal quantities as an anticoagulant. Vitamin C was determined in plasma by the method of Mindlin and Butler (5); and in urine by the method of Evelyn et al. (6). For creatinine, the plasma and urines were precipitated using the ferric sulphate-

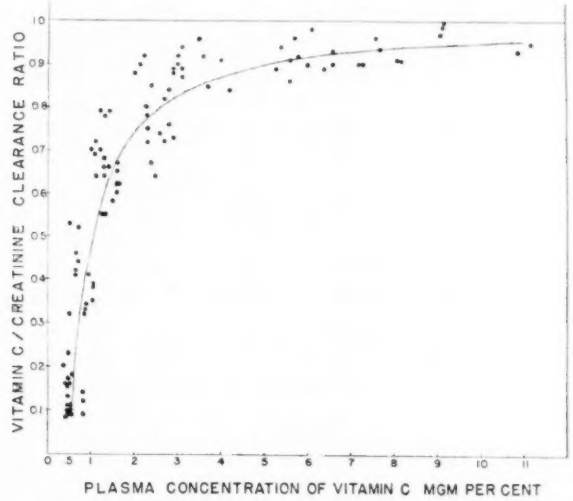

Fig. 1. The relationship between the vitamin C creatinine clearance ratio and the plasma level of vitamin C

barium carbonate method (7) and then determined by the Folin and Wu (1919) (8) method.

Results. In all a total of 102 clearance periods were run on the four normal dogs. The plasma concentration of vitamin C was varied from 0.40 to 10.9 mgm. per cent. The vitamin C/creatinine clearance ratio is plotted against plasma concentration in figure 1. It should be noted that the vitamin C clearance has a low value at low plasma ascorbic acid concentrations. As the plasma concentration was raised there was a sharp rise in the vitamin C clearance which approached the creatinine clearance as a limiting value.

The data were analyzed to determine whether the renal tubules reabsorbed the vitamin up to some maximal limiting rate after which any

vitamin present in the glomerular filtrate would be excreted in the urine. The absolute amount of the vitamin reabsorbed by the tubules was calculated by subtracting the amount excreted per minute from the amount

TABLE 1

Maximal reabsorption of vitamin C

DOG NUMBER	PLASMA CONC.	FILTERED		EXCRETED		REABSORBED	
			Glomerular filtrate		Glomerular filtrate		Glomerular filtrate
	mgm. per cent.	*mgm. per min.*	*mgm. per 100 cc.*	*mgm. per min.*	*mgm. per 100 cc.*	*mgm. per min.*	*mgm. per 100 cc.*
190	0.80	0.39	0.80	0.04	0.08	0.35	0.72
	2.36	1.24	2.36	0.86	1.64	0.38	0.72
	7.36	4.37	7.36	3.95	6.62	0.42	0.74
189	1.05	0.45	1.05	0.17	0.39	0.28	0.66
	1.62	0.83	1.62	0.51	1.00	0.32	0.62
190	0.46	0.21	0.46	0.004	0.01	0.21	0.45
	2.77	1.59	2.77	1.35	2.35	0.24	0.42
211	2.51	1.38	2.51	1.03	1.88	0.35	0.63
	6.49	4.28	6.49	3.90	5.91	0.38	0.58
190	2.77	1.64	2.77	1.21	2.03	0.43	0.74
	5.43	2.91	5.43	2.53	4.72	0.38	0.71

TABLE 2

Effect of hyperglycemia on the reabsorption of vitamin C

PLASMA VITAMIN C	PLASMA GLUCOSE	CLEARANCES			VITAMIN C CREATININE CLEARANCE RATIO	VITAMIN C REABSORBED	
		Vitamin C	Creatinine	Glucose			
mgm. per cent	*mgm. per cent*	*cc. per min.*	*cc. per min.*	*cc. per min.*		*mgm. per min.*	*mgm. per 100 cc. filtrate*
0.90	109	31.6	52.8		0.60	0.19	0.36
0.87	114	22.8	46.1		0.49	0.21	0.46
0.84	120	20.9	47.8		0.44	0.23	0.47
0.68	510	22.2	62.5	30.2	0.36	0.27	0.44
0.68	510	24.8	69.2	40.8	0.36	0.30	0.44
0.68	510	21.0	71.5	33.6	0.29	0.34	0.48

filtered per minute. Assuming that the vitamin was completely filterable from the plasma the latter value was obtained by multiplying the creatinine clearance by the plasma concentration of the vitamin.

Table 1 gives the quantity of vitamin C reabsorbed by the tubules in

33 observations in 3 dogs at 10 different plasma levels, each figure being the average of 3 observations. These data indicate that there is a limitation in the capacity of the renal tubules to reabsorb the vitamin and that it is this circumstance that leads to the increased excretion at elevated plasma levels.

Another experiment was run in order to determine whether glucose and vitamin C were reabsorbed by a common mechanism in the dog. Table 2 gives the results of this test. When the plasma concentration of glucose was raised above 500 mgm. per cent, putting a maximum strain on the glucose reabsorptive system there was no impairment of the reabsorption of vitamin C. From this it may be inferred that the reabsorption of vitamin C and of glucose does not involve a common mechanism.

The data were also analyzed with a view to determining whether or not the rate of urine flow affected the excretion of vitamin C. Absolutely no relationship was found between these two when the urine flow varied from 1.0 to 8.7 cc./minute.

Discussion. The validity of the calculation of the active tubular reabsorption depends on the assumption that the vitamin is completely filterable at the glomerule. Leblond (9) has demonstrated that this circumstance exists in the frog, and our work (1) on the human kidney also supports this viewpoint. The data indicate that vitamin C in the dog, as in man, is excreted only by filtration; that it is actively reabsorbed by the renal tubules, and that the factor which limits this reabsorptive process is the existence of a maximal rate. This phenomenon of a maximal rate of transfer has been shown to obtain for glucose (10) and for vitamin C in man (1) and for the tubular excretion of many organic compounds (4). Shannon (4) has suggested that this limitation may follow from the circumstance that in the process of active transfer the substance enters into reversible combination with some cellular element present in a limited and constant amount and it is the rate of decomposition of this complex which limits the progress of the over-all reaction of transfer. In the case of vitamin C this situation may be represented as follows:

(1) $$\underset{\substack{\text{tubular}\\\text{ascorbic acid}}}{A} + B \rightleftarrows \underset{\substack{\text{cellular}\\\text{complex}}}{AB} \rightarrow \underset{\substack{\text{distal}\\\text{ascorbic acid}}}{Tr} + \underset{\substack{\text{liberated}\\\text{cellular}\\\text{component}}}{B}$$

Applying the law of mass action to such a condition the following relationship (4) may be derived:

(2) $$K = (a - Tr/v)\left(\frac{Tm - Tr}{Tr}\right)$$

where Tm represents the maximal rate of tubular reabsorption, v the rate of glomerular filtration in units of 100 cc., a the plasma concentration, Tr the calculated rate of tubular reabsorption and K a constant.

The smooth curve shown in figure 1 was calculated with the following equation:

(3) Vitamin C/creatinine clearance ratio = 1 − Tr/a

The values for Tr were obtained by using equation 2 in which the value for Tm was 0.52 mgm./100 cc. filtrate (the average maximal rate observed in all our observations); v was equal to 100 cc./mm. and K had a value of 0.01.

The data obtained fit this calculated curve satisfactorily and indicate that the hypothesis upon which equations (1) and (2) are based can be used to describe the reabsorptive system at all plasma concentrations examined in the dog.

Therefore, in the dog, ascorbic acid is excreted by filtration and active tubular reabsorption, and the excretion of vitamin C is dependent upon the plasma concentration of the vitamin, the rate of glomerular filtration, and the maximal rate of tubular reabsorption. This mechanism of the excretion in an animal capable of synthesizing its own ascorbic acid is similar to the mechanism by which man excretes the vitamin (1). However, the ability of the tubules to reabsorb the vitamin in the dog (0.52 mgm./100 cc. filtrate) is much less than that of man, e.g., 1.7 mgm./100 cc. filtrate.

SUMMARY

1. Simultaneous vitamin C and creatinine clearances in the normal dog show that ascorbic acid is excreted by filtration and active tubular reabsorption.

2. The reabsorptive mechanism for vitamin C appears to be limited by a maximal rate so that when the vitamin is presented to the tubules by the glomerular filtrate at a rate exceeding this maximum the excess is excreted in the urine.

3. Vitamin C is not reabsorbed by the same mechanism as glucose.

REFERENCES

(1) Ralli, E. P., G. J. Friedman and S. H. Rubin. J. Clin. Investigation **17:** 765, 1938.
(2) Friedman, G. J., S. Sherry and E. P. Ralli. In press.
(3) Smith, H. W. Personal communication.
(4) Shannon, J. A. Physiol. Reviews **19:** 63, 1939.
(5) Mindlin, R. L. and A. M. Butler. J. Biol. Chem. **122:** 673, 1938.
(6) Evelyn, K. A., H. T. Malloy and C. Rosen. J. Biol. Chem. **126:** 645, 1938.
(7) Steiner, A. F., F. Urban and E. S. West. J. Biol. Chem. **98:** 289, 1932.
(8) Folin, O. and H. Wu. J. Biol. Chem. **38:** 81, 1919.
(9) Leblond, C. P. Compt. rend. Soc. de biol. **127:** 208, 1938.

ACETYLCHOLINE HYDROLYSIS BY THE FROG'S SARTORIUS MUSCLE[1]

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The problem of chemical transmission of motor nerve impulses to voluntary muscle has been approached by Marnay and Nachmansohn (1) by studying the choline esterolytic activity of various portions of the sartorius muscle of the European frog (Rana esculenta). Pezard and May (2) previously had shown that the pelvic one-fifth of this frog's sartorius has few motor nerve endings, while the adjacent or second fifth is richest in nerve endings and the third fifth has somewhat fewer than the second. Marnay and Nachmansohn determined the choline esterase present in these three portions and found that the second fifth has approximately seven times and the third fifth approximately four times the hydrolytic activity of the pelvic fifth. They also found that the sciatic nerve has approximately eight times the esterolytic activity shown by the pelvic fifth of the muscle and concluded that this difference in activity of the various portions of the muscle could not be attributed to the presence of nerve fibers alone, but must be evidence of a concentration of the enzyme at the nerve endings. From their data they calculate that 0.001 μg. of acetylcholine can be hydrolysed at the nerve endings of a sartorius muscle weighing 300 mgm. during the refractory period of the muscle.

Confirmation of this work comes from similar studies on the sartorius of the toad made by Feng and Ting (3) but under different experimental conditions. They found that the pelvic one-fourth has much less choline esterase than the portion of muscle near the nerve entrance.

In view of the discrepancies in experimental conditions between the work of Marnay and Nachmansohn and that of Feng and Ting, it was deemed desirable to obtain data on Rana pipiens, the frog commonly used in this country for experimental work, and some experiments are herewith reported using the procedure of Marnay and Nachmansohn.

METHOD. Sartorius muscles of Rana pipiens were removed and divided as quickly as possible into five equal portions. Those portions repre-

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senting the pelvic, second, and third fifths of the muscles were weighed and ground with quartz powder and Ringer solution to form a suspension of the tissue. This suspension was transferred to volumetric flasks and made to the desired volume with Ringer solution which had the same composition as that used by Marnay and Nachmansohn. After shaking thoroughly each time, 4 cc. portions of this suspension were transferred to Warburg vessels. The side-bulbs contained 0.5 cc. (10 mgm.) of acetylcholine bromide which will give an initial concentration of 1.6 mgm. of acetylcholine in 1 cc. The vessels were equilibrated with a 5 per cent CO_2-N_2 mixture; they were then tipped and the production of CO_2 was followed, in most cases, for 120 minutes. The temperature of the bath was 18°C.

In preliminary experiments no spontaneous hydrolysis of acetylcholine, nor spontaneous production of acid by the tissues, was found under these experimental conditions.

Previous experiments by one of us (4) have shown the necessity of prostigmine or physostigmine controls, since one sometimes gets hydrolysis of acetylcholine which is not due to choline esterase. Such controls in the experiments reported here showed that there was no hydrolysis of the ester by muscle with physostigmine present in a dilution of 1:10,000.

When working with biological fluids or tissue suspensions, buffers other than $NaHCO_3$ are present, and the production of CO_2 is not equivalent to the acetylcholine hydrolysed. This necessitates the addition of an acid retention factor to the vessel constant. Warburg, Kubowitz and Christian (5) have devised an experimental method for determining a composite flask constant (K_M) for serum by adding a known quantity (X_{CO_2}) of lactic acid to the serum in the presence of an excess of $NaHCO_3$ and measuring the CO_2 pressure (h) which is developed. The flask constant for each vessel is calculated from their equation:

$$K_M = \frac{X_{CO_2}}{h} \tag{1}$$

Since in our work varying weights of tissue are used, K_M would not be constant from one experiment to another, and, in order to apply this method to the conditions of our experiments, it has been necessary to break the composite factor into its component parts:

$$K_M = K_{CO_2}^{\text{Ringer}} + \Delta K_M \tag{2}$$

where ΔK_M is the retention factor for the particular weight of tissue used. From equation (2) ΔK_M may be calculated since $K_{CO_2}^{\text{Ringer}}$ is known for each flask and K_M may be experimentally determined. Assuming that

ΔK_M is proportional to the weight (w) of tissue, the retention factor per milligram of tissue ($\Delta' K_M$) may be calculated as follows:

$$\Delta' K_M = \frac{\Delta K_M}{w}. \tag{3}$$

Knowing $\Delta' K_M$ for each vessel, the value of K_M for a particular weight of tissue may be calculated as follows:

$$K_M = K_{CO_2}^{\text{Ringer}} + (w \times \Delta' K_M). \tag{4}$$

From the value of K_M for a particular weight of tissue and vessel, the volume of CO_2 in cubic millimeters produced during the experimental period will follow by rearrangement of equation (1):

$$X_{CO_2} = hK_M. \tag{5}$$

The value of $\Delta' K_M$ for each vessel was determined in several experiments using quantities of tissue which varied from 56 to 116 mgm.

TABLE 1

Values of K_M for 100 mgm. of muscle calculated from experimentally determined values of $\Delta' K_M$

	VESSEL NUMBER						
	2	3	4	5	6	7	WEIGHT OF TISSUE USED
	$K_{CO_2}^{\text{Ringer}}$						
	1.624	1.632	1.573	1.647	1.560	1.543	
							mgm.
K_M	2.014	1.890	1.839	1.857	1.744	1.796	62
	1.932	1.873	1.854	1.935	1.812	1.824	115
	1.887	1.903	1.799	1.903	1.810	1.836	116
	1.961	1.901	1.868	1.935	1.838	1.792	115
	1.896	1.881	1.812	1.889	1.822	1.865	116
	1.929	1.944	1.820	1.912	1.820	1.757	100
	1.926	1.919	1.897	1.858	1.850		56
			1.777		1.764		56

RESULTS. In order that the results obtained from the determinations of the acid retention factor may be compared more easily, the K_M values for 100 mgm. of tissue have been calculated from equation (4) above, using the values of $\Delta' K_M$ which were experimentally determined with different weights of tissue. Those values have been collected in table 1. It will be seen that the K_M values for each flask have a maximum variation of 3.6 to 6.3 per cent. This proves the original assumption that the reten-

tion is proportional to the weight of tissue used if all other factors are kept constant and establishes the validity of the application of this formula in our calculations.

Table 2 summarizes the results obtained on the muscles of forty-seven frogs. The choline esterase activity is expressed, using the nomenclature of Nachmansohn (6), as the milligrams of acetylcholine hydrolysed by 100 mgm. of tissue in 60 minutes ($Q_{Ch.E}$). It will be seen that the second fifth has a rate of hydrolysis which is three times that of the pelvic fifth, and the third fifth has a rate which is a little more than twice that of the pelvic fifth.

The average of four experiments with the frog's sciatic nerve gives a $Q_{Ch.E}$ of 0.677, or nearly seven times that of the pelvic fifth of the sartorius muscle.

TABLE 2

Hydrolysis of acetylcholine by portions of frog's sartorius muscle $Q_{Ch.E}$ = mgm. acetylcholine hydrolysed/100 mgm. muscle/60 min.

NUMBER	PELVIC FIFTH		SECOND FIFTH		THIRD FIFTH	
	Muscle weight	$Q_{Ch.E}$	Muscle weight	$Q_{Ch.E}$	Muscle weight	$Q_{Ch.E}$
	mgm.		*mgm.*		*mgm.*	
1	128	0.076	108	0.221		
2	137	0.081	119	0.317		
3	140	0.100	123	0.328	122	0.220
4	142	0.060	132	0.265	132	0.205
5	165	0.133	141	0.285	123	0.192
6	162	0.157	140	0.391	119	0.267
Average.....		0.101		0.301		0.221

Discussion: The results reported here on Rana pipiens agree qualitatively with those obtained by Marnay and Nachmansohn on Rana esculenta, but they do not agree quantitatively, for Marnay and Nachmansohn found that the $Q_{Ch.E}$ values for the pelvic, second and third fifths of the sartorius for Rana esculenta were 0.135, 0.795 and 0.404 respectively, while the corresponding values for Rana pipiens were 0.101, 0.301 and 0.221, or a difference of 26 per cent, 62 per cent and 45 per cent respectively. There is also a relative difference in the esterolytic activity of the muscles from the two frogs, since the $Q_{Ch.E}$ value for the second fifth of Rana esculenta is approximately seven times that of the pelvic fifth, while the $Q_{Ch.E}$ value for the second fifth of Rana pipiens is three times that of the pelvic fifth; and the value for the third fifth of Rana esculenta is four times that of the pelvic fifth, while for Rana pipiens the value of the third

fifth is a little more than twice that of the pelvic fifth. These differences in the corresponding values for the muscles of Rana esculenta and Rana pipiens suggest that the distribution of motor nerve endings in the two muscles is not the same.

In a later paper (6) Nachmansohn says: "Marnay and Nachmansohn have shown that there exists an extremely high concentration of choline esterase at the nerve endings of voluntary muscle and that during the refractory period of voluntary muscle, in a few milliseconds, the amount of acetylcholine required by the theory of chemical transmission can well be hydrolysed at the nerve endings." The amount of acetylcholine which must be liberated at the nerve ending to satisfy the theory of chemical transmission is not known to us, and, furthermore, the amount of acetylcholine actually liberated at the nerve ending in the sartorius muscle of the frog has not been determined; until these quantities are known it is impossible to say that the choline esterase which is also present can effect its hydrolysis in a few milliseconds.

Clark et al. (7) have shown that the activity of choline esterase depends upon its concentration in solution. Marnay and Nachmansohn assumed a nerve ending volume of $\frac{1}{1000}$ of the muscle volume, and later, in answer to the objections of Clark et al., Nachmansohn (6) assumed a nerve ending volume of 1000 μ^3, in which the enzyme would have a concentration high enough to permit maximum activity. Until proof is presented to the contrary, it appears that it must be assumed that the esterase and acetylcholine are acting in the volume occupied by the motor end-plate of the nerve fiber. From measurements which we have made of the drawings (with magnification given) by Kühne (8) of the end-plates in skeletal muscles of Rana esculenta, it is estimated that this volume is several times more than 1000 μ^3. Until such measurements of volume are accurately made, it is not possible to say that the choline esterase which is present in the end-plate has a concentration sufficiently high to give the enzyme maximum activity.

Although it is evident that choline esterase is concentrated to some extent at the motor nerve ending, one is unable to say yet how effective the enzyme is in hydrolysing the acetylcholine which is liberated there during stimulation.

SUMMARY

1. The method of Warburg, Kubowitz and Christian for calculating the acid retention of serum has been applied to tissue suspensions.

2. The rate of hydrolysis of acetylcholine by the second fifth of the sartorius muscle of Rana pipiens is three times that of the pelvic fifth. The rate of hydrolysis of the third fifth is approximately twice that of the

pelvic fifth. There is an absolute and relative difference, in this respect, between the muscle of Rana pipiens and Rana esculenta, which suggests a difference in the distribution of motor endings in the two cases.

3. It is not yet possible to state that the choline esterase which is present at the motor nerve ending will hydrolyse in the refractory period the acetylcholine which is liberated there.

REFERENCES

(1) MARNAY, A. AND D. NACHMANSOHN. J. Physiol. **92:** 37, 1938.
(2) PEZARD, A. AND R. M. MAY. Compt. rend Soc. Biol. **124:** 942, 1937.
(3) FENG, T. P. AND YEN-CHIEH-TING. Chinese J. Physiol. **13:** 141, 1938.
(4) LITTLE, J. M. Proc. Soc. Exper. Biol. and Med. **42:** 197, 1939.
(5) WARBURG, O., F. KUBOWITZ AND W. CHRISTIAN. Biochem. Ztschr. **242:** 170, 1931.
(6) NACHMANSOHN, D. J. Physiol. **95:** 29, 1939.
(7) CLARK, A. J., J. RAVENTOS, E. STEDMAN AND E. STEDMAN. Quart J. Exper. Physiol. **28: 77**, 1938.
(8) KÜHNE, W. Ztschr. f. Biologie **23:** 1, 1887.

CHEMICAL COMPOSITION OF UTERINE SECRETIONS[1]

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Many studies have been made on the nature of uterine epithelium and the effect of endocrine secretions on it, but no chemical analyses have been reported on the product of these secretory cells, the uterine fluid. The amount of this fluid varies in different species. Long and H. M. Evans (1922) first described distention of the normal rat uterus with fluid which accumulates in pro-oestrus and oestrus, with discharge during late oestrus. Warren (1938) found that about 18 times as much uterine fluid was present in pro-oestrus as in dioestrus; 3 weeks following surgical occlusion of the cornua in rats the average total amount of fluid in the uterus was 5.5 cc. De Marco (1930) using uterine fistulae in dogs found that pilocarpine stimulated the flow of uterine fluid so that 0.5 to 1.75 cc. were obtained per hour. E. I. Evans (1933) found scant amounts of fluid in the canine uterus. Schoekaert and Delrue (1938) determined the pH of the cervical mucus in women.

METHODS. Chemical studies were made on the uterine secretion of 5 dogs, 6 rabbits and 18 rats.

Physiological methods. Ether anesthesia was used for all surgical procedures, which consisted in preparation of uterine fistulae or closed segments; except in the dogs with fistulae, all of the uterine fluids were obtained by opening the abdomen surgically and aspirating the uterus with a fine needle.

In 3 dogs uterine fistulae were made according to the method used by Reynolds (1930) for rabbits, and following healing, freshly prepared pilocarpine hydrochloride, 6 mgm. in saline, was injected intravenously as a secretory stimulant. No fluid was obtained from the cervices unless the dog was in heat, so that stilboestrol 0.1 to 1.0 mgm. was injected intramuscularly in all dogs daily. Subsequently in 2 of these animals a closed uterine segment was prepared by ligation of the uterus proximally and distally. In 2 additional dogs similar closed uterine segments were prepared by proximal and distal ligation of the uterine horns. Fluid was obtained from the closed segments by aspiration at weekly intervals.

[1] This investigation was aided by a grant from the Christian Fenger Memorial Fund.

Similar methods were used in the rabbits and rats, but insufficient fluid was obtained from uterine fistulae for chemical analyses so that secretion was secured from the closed segments in most cases. In these groups uterine secretion was obtained: 1, following uterine ligation alone (2 rabbits, 5 rats); 2, uterine ligation and estrogen injection (4 rabbits, 10 rats); and 3, estrogen injection without ligation (1 rabbit, 5 rats). In 5 rats fluid was removed from the normal uterus during oestrus without preliminary ligation and in 5 rats estrogen was injected beginning 3 weeks following bilateral ovariectomy. As estrogenic material either stilboestrol 0.1 mgm. or estrone 10 I.U. in oil was injected daily.

Chemical methods. Water determinations were made by drying known weights of the fluid to constant weight at 105°C. Chloride was determined by the method of Van Slyke (1923) as modified by Wilson and Ball (1928); sodium, by the method of Butler and Tuthill (1931); calcium, by the method of Kramer and Tisdall (1921) as modified by Clark and Collip (1925); and potassium, by the method of Shohl and Bennett (1928) as modified by Eichelberger (1938). Glucose was determined by the method of Shaffer and Hartman (1921) as modified by Somogyi (1926); inorganic phosphorus, by the method of Fiske and Subbarow (1925). Total nitrogen and non-protein nitrogen were determined by the micro-Kjeldahl method of Koch and McMeekin (1924) and the proteins by multiplying by 6.25 the total nitrogen corrected for non-protein nitrogen. pH was determined with the glass electrode and the total CO_2 by the method of Van Slyke and Neill (1924) on fluid collected under oil.

Results and discussion. Uterine fluid was found to be limpid, slightly opalescent and contained occasional epithelial cells on microscopic examination. As compared with prostatic secretion obtained by pilocarpine stimulation after isolation of the prostate gland according to the technique of Huggins, Masina, Eichelberger and Wharton (1939), the fluid was scant in amount. The rate of secretion in dogs with uterine fistulae after pilocarpine stimulation was 0.2 to 0.4 cc. per hour; thus only several chemical determinations were made on the fluid obtained at any one sitting of a few hours' duration.

In animals with closed uterine segments, larger quantities of fluid were obtained. The volume obtained from the 2 horns of the uterus of the rat varied from 0.2 to 1.4 cc.; of the rabbit, from 1 to 5.5 cc.; and from 7 to 17 cc. in the dog.

The chemical composition of uterine fluids is shown in table 1. Points of similarity in the fluids from the dog, rabbit and rat uteri were the high content of water and sodium and the low protein content. Phosphate and glucose were present in very small amounts or absent.

The uterine fluids of rat and rabbit resembled each other and were characterized by an alkaline pH, a high total CO_2 content, with the chloride

concentration being normal with respect to plasma. The uterine fluid of the dog differed from these in having an acid pH, a low total CO_2 content and a high chloride concentration.

TABLE 1

Chemical composition of uterine fluid

	H_2O	pH	CO_2	TOTAL N	NPN	PROTEIN	Cl	Na	Ca	K	GLUCOSE	INORGANIC P
Dog												
	gm.		*mM*	*gm.*	*gm.*	*gm.*	*mM*	*mM*	*mM*	*mM*	*mgm.*	*mM*
1†	986			0.30	0.19	0.70	180	170	4.7	4.1	0	0
	985			0.40	0.13	1.72	190	140	2.8	6.4	80	0
2†	986	6.21	3.0	0.50	0.30	1.25	149	165			0	0
3‡	975	6.26	3.8		0.11		149	164				
4‡	986	5.91	2.6	1.09	0.27	5.12	156	169			70	0.03
5‡	986	6.05	2.6	1.76	0.17	9.94	157	161			70	0.03
Average*	984	6.09	3.0	0.8	0.20	3.8	167	162	3.5	5.2	0–80	0–0.03
Rabbit												
Uterine ligation	983	7.73	55.3	0.67	0.44	1.41	91	166	7.9	6.1	0	0
	981	7.80	48.2	0.94	0.29	4.0	99	150	1.5		160	0.14
Estrone	980	7.90					98	191				
	976	7.73	51.2									
Uterine ligation & estrone	976	7.75	48.4				103				100	0.20
Average*	979	7.78	53.6	0.8	0.37	2.7	98	158	4.7	6.1	0–160	0–0.20
Rat												
Stilboestrol injections	985	7.68					106	215				
Uterine ligation & stilboestrol	979	7.66	61.8	1.32	0.29	6.53	101	123	1.5	4.3	150	0
				0.47		1.12	98					
Ovariectomy and estrone	990	7.32					92					0
Average*	982	7.55	61.8	1.0	0.29	5.1	98	169	1.5	4.3	150	0

Values expressed per liter of fluid.

* Averages represent all of our data, not merely those represented in this table.

† Uterine fistula, pilocarpine stimulation.

‡ Uterine ligation, pilocarpine not used.

The distribution of electrolytes in uterine fluids is thus different from that in serum and transudates, demonstrating that it is formed by the process of secretion. The low concentration of protein requires readjustments to be made to secure osmotic equilibrium. In all the animals sodium

was the cation present in largest amounts; the principal anion in fluid from the dog was chloride, bicarbonate being low; while in rat and rabbit the chloride was at serum level and the bicarbonate concentration was high.

It is noteworthy that the various procedures employed resulted in no differences in chemical composition in the same species and especially that the fluid obtained after stimulation with pilocarpine in dogs with uterine fistulae resembled that in the closed segment without drug injection.

The similarity of rat and rabbit uterine fluids has been discussed above. The uterine fluid of the dog, differing from these, resembles closely, however, certain fluids of the genital tract in the male, notably prostatic fluid obtained after pilocarpine stimulation in dogs and spermatocele fluid obtained from the epididymis in man (table 2). Thus, there are recurrent

TABLE 2

Comparison of chemical composition of genital tract fluids

	H_2O	pH	CO_2	TOTAL N	NPN	PROTEIN	Cl	Na	Ca	K	GLUCOSE	INORGANIC P
	gm.		*mM*	*gm.*	*gm.*	*gm.*	*mM*	*mM*	*mM*	*mM*	*mgm.*	*mM*
Rabbit uterine fluid	979	7.78	53.6	0.8	0.37	2.7	98	158	4.7	6.1	0–160	0–0.20
Rat uterine fluid	982	7.55	61.8	1.0	0.29	5.1	98	169	1.5	4.3	0–150	0
Dog uterine fluid	984	6.09	3.0	0.8	0.20	3.8	167	162	3.5	5.2	0–80	0–0.03
Dog prostatic fluid*	981	6.16	0.8		0.22	8.3	160	159	0.3	5.1	0–300	
Human spermatocele fluid†		7.13	6.3		0.26	5.5	139		2.3		0–tr.	0

Values expressed per liter of fluid.

* Following pilocarpine stimulation: data of Huggins, Masina, Eichelberger and Wharton (1939).

† Data of Huggins and Johnson (1933).

regional chemical patterns in the fluids of the genital tract, one pattern seen in male and female dogs and the male human individual, while another is found in female rats and rabbits. The low content of glucose and phosphate in uterine fluids in the dog, rat and rabbit, in the prostatic fluid of dog and man, and in spermatocele fluid in man must be sharply contrasted with the high phosphate and glucose content of the human seminal vesicle first described by Huggins and Johnson (1933).

In the rat the uterine horns were found to be atrophic and collapsed 3 weeks after bilateral ovariectomy and became distended after estrogenic stimulation for 1 week. Analyses of the fluid showed it to be essentially the same as that in rats which had not been spayed or injected with estrone, demonstrating that estrogen alone as a hormone can produce in the uterus this characteristic fluid.

CONCLUSIONS

Uterine fluids obtained from rats and rabbits resemble each other chemically. They have points of similarity to and difference from uterine fluid of the dog. The points of similarity are the high content of water and low concentrations of protein, glucose and inorganic phosphate; the principal cation is sodium. The fluids differ in that in the dog the pH is acid, total CO_2 content is low and chloride relatively high, while the uterine fluid of rats and rabbits is characterized by an alkaline pH, high total CO_2 content, with the chloride concentration at the plasma level.

No significant differences in chemical composition were detected in fluid obtained by pilocarpine stimulation from uterine fistulae or in closed segments without stimulation. Injections of estrogen alone in spayed rats produced the uterine fluid typical of the normal rat.

Certain chemical patterns are seen in the similarity of fluids from various regions of the genital tracts of male and female animals. Thus rat and rabbit uterine fluids are identical from an electrolyte standpoint and differ from dog uterine fluid, dog prostatic fluid obtained after pilocarpine stimulation and human spermatocele fluid, the last three resembling each other.

REFERENCES

Butler, A. M. and E. Tuthill. J. Biol. Chem. **93:** 171, 1931.
Clark, E. P. and J. B. Collip. Ibid. **63:** 461, 1925.
De Marco, R. Arch. di Fisiol. **29:** 181, 1930.
Eichelberger, L. J. Urol. **40:** 366, 1938.
Evans, E. I. This Journal **105:** 287, 1933.
Fiske, C. H. and Y. Subbarow. J. Biol. Chem. **66:** 375, 1925.
Huggins, C. and A. A. Johnson. This Journal **103:** 574, 1933.
Huggins, C., M. H. Masina, L. Eichelberger and J. D. Wharton. J. Exper. Med. **70:** 543, 1939.
Koch, F. C. and T. L. McMeekin. J. Am. Chem. Soc. **46:** 2066, 1924.
Kramer, B. and F. F. Tisdall. J. Biol. Chem. **48:** 223, 1921.
Long, J. A. and H. M. Evans. Mem. Univ. Calif. **6:** 24, 1922.
Reynolds, S. R. M. This Journal **92:** 420, 1930.
Schockaert, J. A. and G. Delrue. Bull. Acad. roy. med. de Belgic **3:** 601, 1938.
Shaffer, P. A. and A. F. Hartman. J. Biol. Chem. **45:** 349, 1921.
Shohl, A. T. and H. B. Bennett. Ibid. **78:** 643, 1928.
Somogyi, M. Ibid. **70:** 599, 1926.
Van Slyke, D. D. Ibid. **58:** 523, 1923.
Van Slyke, D. D. and J. M. Neill. Ibid. **61:** 523, 1924.
Warren, M. R. This Journal **122:** 602, 1938.
Wilson, D. W. and E. G. Ball. J. Biol. Chem. **79:** 221, 1928.

EFFECTS OF MAGNESIUM ON THE NERVOUS SYSTEM IN RELATION TO ITS CONCENTRATION IN SERUM[1,2]

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A series of cardiovascular changes during the slow intravenous injection of magnesium salts has been previously described (11, 13), each event appearing during a characteristic range of concentration of magnesium in the serum. The relation between concentration on the one hand and changes in reflex activity and in neuromuscular transmission on the other forms the subject of the present study.

METHODS. Decerebrate cats were usually employed, with supplementary experiments on neuromuscular transmission in animals anesthetized with sodium amytal. The muscles to be studied were isolated by appropriate nerve and tendon section and the contraction recorded with a torsion wire myograph. The leg was held rigidly by drills fixed in the bone. A thyratron stimulator was used to give single or repetitive supramaximal break shocks at various frequencies. Magnesium sulfate in isotonic solution was injected slowly into the femoral vein at a constant rate. Samples of serum obtained at intervals were analyzed for magnesium by a method previously described (14). Concentrations corresponding to particular effects were obtained by interpolation between determined values. In some experiments the ureters were tied to prevent urinary excretion. Artificial respiration was employed in all experiments after natural respiration failed and throughout the experiment when the thorax was opened.

RESULTS. The results of the entire series of experiments are summarized in table 1. The figures in parentheses represent individual values in different experiments. In figure 1 the concentrations at which repetitive shocks to the motor nerve just failed to produce a response are plotted against frequency of stimulation.

Single twitches disappeared at concentrations ranging from 6 to 14 mEq

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per liter. At this level the muscle still responded to even the slowest tetanic stimuli. Twitches obtained from the quadriceps, the gastrocnemius-soleus and from isolated strips of diaphragm all gave similar values. In experiments in which two of these muscles were stimulated simultaneously the twitches disappeared at the same time in both muscles. Concentrations of magnesium sufficient to induce failure of response to tetanic stimuli (fig. 1) varied with the frequency of stimulation, fast stimuli still being effective at concentrations far above those at which slower stimuli had ceased to act. Tetani of a given frequency failed in all different muscles at the same time, just as did the twitches.

Two special characteristics of partial neuromuscular block were noted. 1. With partial neuromuscular block due to magnesium the characteristic

TABLE 1

The concentration of serum magnesium required for extinction of neuromuscular transmission and certain reflexes

CONCENTRATION OF MAGNESIUM IN SERUM	FAILURE OF RESPONSE TO STIMULATION OF MOTOR NERVE, RATE OF STIMULATION	FAILURE OF REFLEX
*mEq per l.**		
6–14	Single twitches	Tendon jerks (8, 10, 12, 13)
12–15	Tetani at 20 per sec.	Crossed extensor and shortening reflexes (8, 14, 22)
15–20	Tetani at 40 per sec.	Natural respiration (17, 19, 20, 21, 22)
20–25	Tetani at 60 per sec.	
25–30	Tetani at 100 per sec.	Vibrissa and prima reflexes (20, 26, 27, 29)
30–35	Tetani at 200 per sec.	Corneal reflex (29, 30, 32)

* In each experiment the sequence of neuromuscular and reflex failure given in this table took place as progressively higher levels of serum magnesium were attained. The overlapping of serum magnesium values is due to variations in the absolute level in the several experiments.

self inhibition ("Wedensky" inhibition) of the tetanus appeared at lower frequencies and was more pronounced (figs. 2A, 2B). 2. Twitches which had previously been extinguished reappeared for a time following a tetanus. This "enhancement" or "post-tetanic potentiation" is illustrated in figure 2B.

In every case reflexes were studied simultaneously with the neuromuscular twitches and tetani. Therefore the point of extinction of each reflex was related not only to the serum magnesium value but also to the cessation of neuromuscular activity in each individual experiment. The concentrations were scattered over a considerable range. The knee jerk and other tendon jerks were the first reflexes to disappear, their failure coinciding with that of the neuromuscular twitches. A tetanic contraction

just after the knee jerks had disappeared caused them to reappear for a time, just as it caused the reappearance of direct neuromuscular twitches. No changes in other reflexes could be detected at this level. The crossed-extensor and shortening reflexes failed at somewhat higher concentrations,

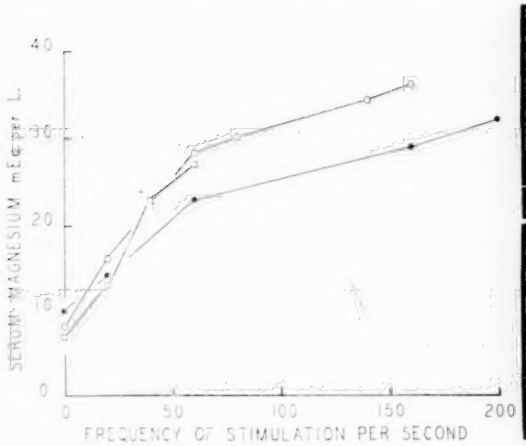

Fig. 1

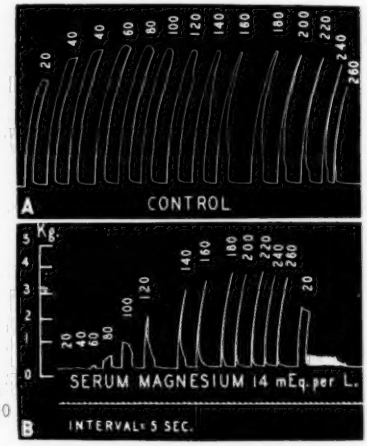

Fig. 2

Fig. 1. The concentrations of magnesium at which tetanic contraction disappeared are plotted against the frequency of stimulation in 3 individual experiments. The more rapid the stimulation, the higher is the concentration of magnesium necessary to block a response. The values at *0* are those for single twitches.

Fig. 2A. Figure 2A represents a control experiment in which no magnesium had been injected. Each number indicates the frequency of stimulation of the tetanic contraction recorded just below it. Self inhibition, or "Wedensky" inhibition, occurs first at 60 per second. The interval between the initial contraction and the onset of inhibition becomes progressively shorter as the frequency of stimulation increases from 60 to 260 per second.

Fig. 2B is taken from the same experiment after magnesium had been injected until the concentration in serum attained 14 mEq per liter. Each numeral indicates the frequency of stimulation of the tetanic contraction recorded below it. Ordinates represent the tension developed by the muscle. Single shocks and slower frequencies of stimulation elicit very little response, and at higher frequencies Wedensky inhibition is much more marked than in the control. At the extreme right is the record of the tetanic response to a stimulus at 20 per second at the close of the experiment. This tetanus was followed for a time by a return of the response to single stimuli, *i.e.*, by "post-tetanic enhancement."

corresponding to the level of failure of slow neuromuscular tetani (frequency of about 20 per second). At a little higher concentration natural respiration failed; simultaneously neuromuscular tetani at the rate of 30 to 50 per second failed. The corneal reflex, the pinna reflex and the

vibrissa reflex (protrusion of vibrissae following stimulation of the ear) all persisted long after respiratory failure, disappearing only when tetani at 60 to 100 per second failed.

It was found that reflexes which had disappeared could be brought back for a time if a brief neuromuscular tetanus were applied to the motor element of the arc. This is of course the same "enhancement" as that previously noted as characteristic of partial neuromuscular block. The height of contraction of muscles directly stimulated was invariably found to be unaffected by magnesium injection.

Discussion. The sequence in which the reflexes disappear (table 1) is quite different from that following the administration of a volatile anesthetic or a barbiturate. With these anesthetics the knee jerk is especially resistant, while the corneal reflex disappears relatively early. Respiration, which disappears so early after magnesium, disappears late in the course of the action of the ordinary anesthetics (8). This difference in effect on reflexes makes it probable that the locus of action of magnesium differs from that of most anesthetics.

The order of disappearance of reflexes after magnesium is, however, correlated with the type and rate of discharge characteristic of the motor neurones comprising the motor element of the reflex arc. The reflexes with slow frequencies of discharge disappear first, those with higher frequencies later. Thus the knee jerk and other tendon reflexes are largely single twitches, each twitch being produced by a single discharge in most of the motor neurones taking part in the reflex (7, 10). The flexion reflex, another twitch-like reflex (5, 7) is known to be abolished at levels of serum magnesium comparable with those reported here for twitches and tendon jerks (3). The crossed-extensor reflex and the shortening reaction in quadriceps or soleus is largely mediated by rhythmic discharges at rates varying from 10 to 20 per second (2, 5, 6, 10). Respiration is mediated in the diaphragm by impulses from phrenic motor neurones discharging 25 to 30 times per second (1), with normal breathing, somewhat more rapidly with forced breathing. The rate of impulses in muscles of the eye, ear and vibrissae is not known, but there is indirect evidence that they may be 100 or more per second (4).

If these characteristic rates of discharge of the different reflexes are compared with the concentrations of magnesium at which neuromuscular tetani of the same rate fail (table 1), an excellent correlation is disclosed. Thus twitch-like reflexes fail when twitches fail, slow tetanic reflexes fail with slow neuromuscular tetani, and rapid tetanic reflexes and rapid neuromuscular tetani disappear together when much higher levels are attained. The suggestion is clear that reflexes fail because of neuromuscular block rather than because of an action of magnesium on the central nervous system. This interpretation is strengthened by the observation that

twitch-like reflexes may be made to reappear by neuromuscular tetani of their motor elements. If failure of the reflexes were due primarily to block at the central synapse, no procedure affecting only the peripheral neuromuscular mechanism could possibly restore the reflex.

Experiments such as these do not in any way exclude an effect of magnesium on the central nervous system. The demonstration by Peck and Meltzer (12) that anesthesia may be produced in man by the injection of magnesium is unequivocal evidence that such a central effect does exist. They do, however, indicate that magnesium affects muscular activity mainly through its action on the neuromuscular junction and not through its action on the central nervous system.

The possibility must be considered that the curare-like action of magnesium excess is in some way the converse of the tetany of magnesium deficiency. The tetany of animals depleted of magnesium is peculiar because it is not necessarily associated with low magnesium in serum, is not immediately relieved by injection of magnesium and is not prevented by curare (9). Failure to abolish these convulsive seizures by curare places the site of disturbance in the muscles themselves, beyond the myoneural junction. Since the response of muscles stimulated directly rather than through their motor nerves is unaffected by excess of magnesium, the locus of action of magnesium excess must be the myoneural junction itself. This difference in point of action suggests that the phenomena of magnesium excess and those of magnesium deprivation are not intimately related.

The conclusions drawn in this study are not in agreement with those of Bryant, Lehmann and Knoefel (3). These authors, using the spinal dog, found that the flexor response to single induction shocks failed while the skeletal muscle still responded to stimulation of its motor nerve. From this observation they concluded that the action of magnesium is primarily central. Our experiments show that this conclusion would be justified only if the type and rate of stimulation of the skeletal muscle is identical with that produced by the natural reflex, since the level of extinction of response to neuromuscular stimulation varies so much with frequency of stimulation. These workers do not specify the type of stimulation used to stimulate the skeletal muscle, so it is not possible to decide whether or not this is actually the cause of the apparent discrepancy between their results and ours.

CONCLUSIONS

1. Concentrations of magnesium at which neuromuscular transmission failed and concentrations at which various reflexes disappeared were determined in the cat.

2. The greater the frequency of stimulation, the higher is the concentration of magnesium necessary to block transmission.

3. It is probable that magnesium affects muscular activity by blocking neuromuscular transmission rather than by means of its depressant action on the central nervous system.

REFERENCES

(1) Adrian, E. D. and D. W. Bronk. J. Physiol. **66:** 81, 1928.
(2) Adrian, E. D. and D. W. Bronk. J. Physiol. **67:** 119, 1929.
(3) Bryant, G. W., G. Lehmann and P. K. Knoefel. J. Pharmacol. and Exper. Therap. **65:** 318, 1939.
(4) Cooper, S. and J. C. Eccles. J. Physiol. **69:** 377, 1930.
(5) Creed, R. S., D. Denny-Brown, J. C. Eccles, E. G. T. Liddell and C. S. Sherrington. Reflex activity of the spinal cord, 1932.
(6) Eccles, J. C. and H. E. Hoff. Proc. Roy. Soc. London **110B:** 483, 1932.
(7) Eccles, J. C. and C. S. Sherrington. Proc. Roy. Soc. London **107B:** 511, 1931.
(8) Forbes, A. and R. H. Miller. This Journal **40:** 148, 1916.
(9) Greenberg, D. M. and E. V. Tufts. This Journal **121:** 416, 1938.
(10) Hoff, E. C., H. E. Hoff and D. Sheehan. J. Physiol. **83:** 185, 1934.
(11) Hoff, H. E., P. K. Smith and A. W. Winkler. This Journal **127:** 722, 1939.
(12) Peck, C. H. and S. J. Meltzer. J.A.M.A. **67:** 1131, 1916.
(13) Smith, P. K., A. W. Winkler and H. E. Hoff. This Journal **126:** 720, 1939.
(14) Smith, P. K., A. W. Winkler and B. M. Schwartz. J. Biol. Chem. **129:** 51, 1939.

COMPARATIVE PARENTERAL AND ORAL ASSAYS OF ADRENAL CORTICAL HORMONE SUBSTANCES

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An early report of the oral treatment of adrenal insufficiency with adrenal cortex was given by Osler in 1896 (1). He apparently successfully treated a case of Addison's disease with a glycerol extract of pig adrenals. With the preparation of epinephrine-free biologically active extracts of the adrenal cortex, adrenal insufficiency has for the past several years been quite commonly treated by parenteral administration. Grollman (2) reported the successful treatment of adrenal insufficiency in rats, also, by the oral administration of a charcoal adsorbate.

In a previous report (3) we described a rat method of assaying adrenal cortex extracts and noted that adrenalectomized rats could be successfully maintained by administration of the extracts in the drinking water, and that such administration gave better growth response than subcutaneous injections. Later reports (4, 5) present further evidence that the adrenalectomized rat is a suitable test animal for the assay of adrenal cortical extracts, and that these extracts, orally, are capable of maintaining the test animal, as Thorn et al. (6) have also found for the adrenalectomized dog. In one of these reports (5) an "oral rat unit" is described.

Recent clinical reports (7, 8) have called attention to the effectiveness in the human subject of orally administered adrenal cortical hormone preparations. Some clinical data (7) indicate that, on the basis of gland equivalence, the administration of the oral preparation is more effective than parenteral injection of the aqueous sterile solution. A study of parenteral and oral assays of adrenal cortical hormone material is therefore desirable.

The present study is concerned with a direct comparison of parenteral and oral assays of adrenal cortex concentrates in the 4-weeks-old adrenalectomized white rat. The comparative effectiveness of the more active crystalline substances of the adrenal cortex, corticosterone, dehydrocorticosterone, and desoxycorticosterone, has also been studied, and the cortical-hormone activity in rat units quantitatively determined by subcutaneous injection, as well as by administration by stomach tube.

EXPERIMENTAL. The composite extracts of the adrenal cortex which were used and which so far as we know contain all the cortical hormone

active substances present in the gland, were prepared from beef suprarenal glands by the process previously reported (9).

For the determination of the parenteral and oral activity of desoxycorticosterone acetate, the synthetic material was used. The corticosterone and dehydrocorticosterone were obtained from the least water-soluble fraction of a gland extract by procedures previously described (10). They were isolated in pure form and identified by their melting points, analyses, and specific rotations.

METHODS. The composite extracts were assayed parenterally as previously described (3). For the determination of their oral effectiveness they were assayed both by administration in the drinking water and by administration by stomach tube. For assay by drinking water administration, the daily dose of hormone was added to the estimated amount of water the rat would drink over a 24 hour period. All assays were carried out over a 20 day period, hormone administration being withdrawn on the 20th day. The rat units per cubic centimeter of extract were estimated as described in the previous report, one rat unit being the minimum daily dose of hormone which, administered daily for 20 days to 4-weeks-old male rats weighing 50 to 60 grams, is sufficient to protect at least 80 per cent of the rats and produce an average growth of at least 20 grams for the 20 day period.

Since administration in the drinking water depends on the voluntary consumption by the assay animal, it might be objected that quantitative results can not be obtained, and that a "minimum daily dose" can not therefore be accurately determined. We have, therefore, also carried out assays by administering the extracts once daily by stomach tube. Since in determining the rat unit parenterally one daily subcutaneous injection is given, the most comparable oral determination of rat units can be obtained by one daily administration by stomach tube. We find that a small gauge soft rubber catheter can easily be pushed down a small rat's esophagus, and that oral administration can thus be as easily carried out as subcutaneous injections.

The care of the rats, the diet, and the technique of removal of the adrenal glands are the same as previously described (3).

RESULTS. In table 1 is given a summary of the assays of several adrenal cortex extracts. It can readily be seen that, whether the extracts are assayed parenterally, by subcutaneous injection, orally, by either stomach tube administration or drinking water administration, the result in rat units per cubic centimeter of extract is the same. Adrenal insufficiency in the young adrenalectomized rat can therefore be controlled equally as well by oral administration of cortical extracts as by parenteral administration. For each assay in table 2 at least 10 rats were used. The routine procedure was to administer 0.3 and 0.4 cc. of the extracts per

rat per day for the 20 day period. In some cases 0.2 cc. doses were also given. If an extract failed to pass the test at 0.3 cc., but passed at 0.4 cc., it is reported as having 3.0 rat units per cc., the average of 3.3 and 2.5 being practically 3. If the extract passed well at both doses, 0.3 cc. and 0.4 cc., it is reported to have 4.0, the average of 5 and 3.3, rat units per cc. Nearly all extracts failed to pass the test at 0.2 cc. per rat per day, except in a few instances (51–5 and 54–5). Any extract which barely passed the test at 0.3 cc. is reported as containing 3.3 rat units per cc.

In table 2 are given the results of the parenteral assays of the pure crystalline substances, corticosterone, dehydrocorticosterone, and desoxycorticosterone. The substances were administered in olive oil solution,

TABLE 1

Comparison of parenteral and oral assays of adrenal cortex extracts

PREPARATION	PARENTERAL ASSAYS	ORAL ASSAYS, DRINKING WATER ADMINISTRATION	STOMACH TUBE ADMINISTRATION
	rat units per cc.	*rat units per cc.*	*rat units per cc.*
51–5	5.0	5.0	
54–5	4.0	5.0	
47–5	4.0	<5.0	
288–5	3.3	4.0	4.0
43A–6	3.0	3.0	
44–6	4.0	4.0	
45A–6	3.0	3.0	3.3
48–6	3.0	3.0	
52–6	4.0	4.0	
55–6	3.0	3.0	
291–6	4.0	4.0	4.0
9–6	3.0	3.0	3.3
13–6	4.0	4.0	4.0
29–6	3.0		4.0
292–7	3.0		3.0
66A–6	4.0	4.0	

and the concentrations were such that at all dosage levels 0.2 cc. of the oil solution was injected. It is apparent from the tabulated assay results that, parenterally, corticosterone assays 6 rat units per mgm., while according to our available data dehydrocorticosterone assays about 4 rat units per mgm. Desoxycorticosterone acetate, however, assays 35 rat units per mgm. by subcutaneous injection.

In table 3 are tabulated the results of the oral assays of the pure substances. For these oral assays the substances were also dissolved in olive oil and again the concentrations were adjusted so that the one volume of 0.2 cc. was administered at all dosage levels. As with the composite crude extracts, so with corticosterone, the parenteral and oral assays in

rat units are the same. Dehydrocorticosterone apparently is somewhat more active orally than by injection, assaying orally 6 rat units per mgm. and parenterally 4 rat units per mgm. Desoxycorticosterone acetate, however, differs very strikingly with respect to oral effectiveness. Whereas parenterally the substance assays 35 rat units per mgm., orally it fails to

TABLE 2

Parenteral assays of crystalline adrenal cortex substances

SUBSTANCE	DAILY DOSE	NUMBER OF RATS INJECTED	NUMBER OF RATS SURVIVING 20 DAYS	AVERAGE GROWTH IN 20 DAYS	AVERAGE SURVIVAL AFTER LAST INJECTION	RAT UNITS PER MGM. OF SUBSTANCE
	mgm.			*grams*	*days*	
Corticosterone	0.25	5	5	29	4.0	>4 } 6
	0.125	5	2	13.5	2.0	<8 }
Dehydrocortico-sterone	0.5	3	2	29	6.5	>2 }
	0.25	5	4	21	4.0	4 } 4
	0.125	3	0			<8 }
Desoxycortico-sterone acetate	0.05	5	5	34	5.0	>20 }
	0.033	5	5	34	6.2	>30 } 35
	0.025	5	3	30	8.0	<40 }

TABLE 3

Oral assays of crystalline adrenal cortex substances

SUBSTANCE	DAILY DOSE	NUMBER OF RATS INJECTED	NUMBER OF RATS SURVIVING 20 DAYS	AVERAGE GROWTH IN 20 DAYS OF 20 DAY SURVIVALS	AVERAGE SURVIVAL AFTER LAST INJECTION	RAT UNITS PER MGM. OF SUBSTANCE
	mgm.			*grams*	*days*	
Corticosterone	0.25	9	9	26	7.0	>4 } 6
	0.125	5	0			<8 }
Dehydrocortico-sterone	0.25	5	5	25	6.2	>4 } 6
	0.125	5	0			<8 }
Desoxycortico-sterone acetate	0.033	5	0			<30
	0.10	5	0			<10
	0.20	5	0			<5
	1.0	5	3	11	6	<1

pass the test even at 1 rat unit per mgm. This substance is therefore less than one-thirty-fifth as effective orally as parenterally in the young adrenalectomized rat. This animal therefore can completely utilize orally administered corticosterone, while desoxycorticosterone acetate administered orally can scarcely be used at all. This is very interesting in view of the fact that the substances are so nearly alike chemically.

To determine whether desoxycorticosterone is excreted in the feces and is therefore unabsorbed, the fecal material of the 5 rats that received 1 mgm. daily of the synthetic by stomach tube was collected for one week, from the 7th to the 14th day, and extracted with acetone. The acetone soluble material was dissolved in ether, and the ether solution washed first with dilute HCl, then with 1 per cent Na_2CO_3 solution, and finally with water, after which it was dried with anhydrous $MgSO_4$. The ether soluble substance was transferred to 22 cc. olive oil and the solution injected subcutaneously at 0.2 cc. per rat per day into a group of 5 adrenalectomized rats. The average survival period of this group of 5 rats was 7.8 days as compared with 7 days for a control group. There was therefore no adrenal cortical hormone activity in this fecal extract, and since the desoxycorticosterone acetate assayed 35 rat units per mgm. when administered subcutaneously, there certainly must have been excreted less than 3.3 mgm. of the substance by the entire group of 5 rats for the 7 day period. Since 35 mgm. had been administered over this same period, it would appear that most of the substance was destroyed in the gut.

Discussion of results. Difficulties of oral administration by mixing extracts with the food have been pointed out (5). Since mixing the daily dosage with the drinking water or food makes the hormone intake too dependent "upon the voluntary consumption of food by the assay animal," we have administered the extract by stomach tube. By such single daily oral administration the minimum requirement of the composite gland extract is the same as by single daily subcutaneous injection. The rat unit as previously defined by us[1] can therefore also be used to express adrenal cortical hormone activity by oral assay. It only becomes necessary when stating rat units of a certain extract or substance to designate whether the substance was assayed orally or parenterally, since we know of at least one single pure substance which is less active orally than parenterally.

According to Grollman (11), 1 mgm. of desoxycorticosterone acetate per rat per day, orally or parenterally, is effective in maintaining normal growth in adrenalectomized rats. Our data do not support such a conclusion. According to our rat method of assay, this synthetic substance is less than one-thirty-fifth as active orally as parenterally. It was found orally to assay even less than 1 rat unit per mgm. DeFremery and Spanhoff (12) have also reported that orally, "negative reactions only were obtained with even as much as 500 gamma daily." In this respect, therefore, the synthetic substance differs markedly not only from the natural

[1] A rat unit is defined as the minimum daily dose of hormone which, administered daily for 20 days to 4-weeks-old rats weighing 50 to 60 grams, is sufficient to protect at least 80 per cent of the rats and produce an average growth of at least 20 grams per rat for the 20 day period.

extract, which is just as active orally as by injection, but also from the pure substances, corticosterone and dehydrocorticosterone, which are at least as active orally as parenterally.

SUMMARY

A method of assaying adrenal cortex extracts by stomach tube administration is described and it was found that by oral administration, the gland extracts assayed the same as by parenteral administration.

Corticosterone and dehydrocorticosterone are also as effective orally as parenterally.

Desoxycorticosterone assayed 35 rat units per mgm. parenterally by subcutaneous injection, but orally this substance assayed less than 1 rat unit per mgm.

Cortical hormone activity could not be demonstrated in the feces of the rats that had received desoxycorticosterone acetate by mouth. It appears, therefore, that this substance is destroyed in the gut.

REFERENCES

(1) Osler, W. Internat. Med. Magazine, February, **4:** 1896.
(2) Grollman, A. The adrenals. The Williams and Wilkins Co., Baltimore, 1936.
(3) Cartland, G. F. and M. H. Kuizenga. This Journal **117:** 678, 1936.
(4) D'Amour, F. E. and D. Funk. J. Pharmacol. and Exper. Therap. **62:** 307, 1938.
(5) Blanchard, E. W. and R. C. Tallman. This Journal **124:** 583, 1938.
(6) Thorn, G. W., E. Kendall, Jr. and H. Eisenberg. Endocrinology **23:** 403, 1938.
(7) Conn, J. W. and F. W. Silverman. J. Clin. Investigation **18:** 488, 1939.
(8) Gordon, E. S., E. L. Sevringhaus and M. E. Stark. Endocrinology **22:** 45, 1938.
(9) Cartland, G. F. and M. H. Kuizenga. J. Biol. Chem. **116:** 57, 1936.
(10) Kuizenga, M. H. and G. F. Cartland. Endocrinology **24:** 526, 1939.
(11) Grollman, A. J. Pharmacol. and Exper. Therap. **67:** 257, 1939.
(12) DeFremery, P. and R. W. Spanhoff. Acta Brevia. Neerland., Physiol., Pharmacol., Microbiol. **9:** 79, 1939.

A NOTE ON THE QUESTION OF REFLEX ACTIVATION OF DORSAL ROOT DILATORS

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Since Bayliss (1902) published his observations on the activation of the dorsal root dilators to the legs by stimulation of the depressor nerve, the explanation and indeed the very existence of the phenomenon have been extensively debated by anatomists and physiologists. A review of the literature has recently been presented by Hinsey (1939). Reflex increases in the volume of a sympathectomized but otherwise intact leg of the dog have been reported by Fofanow and Tschalussow (1913) and by Tournade and Malméjac (1933). Rosenblueth and Cannon (1934), though they emphasize that reflex changes in the blood pressure of sympathectomized animals may be explained on the assumption that the dorsal roots are capable of reflex activation, were unable to demonstrate changes in limb volume which would account for the alterations in blood pressure. Downman et al. (1939) report that the vasomotor responses of cats' paws, recorded optically from plethysmographs, are abolished by sympathectomy. The evidence derived from manometric determinations of blood flow is divided between that of Bacq, Brouha and Heymans (1933) and of Derom and Grimson (1939), who obtained negative results, and that of Bishop, Heinbecker and O'Leary (1933), who, with a rather more intricate technique, obtained changes attributed by them to activation of dorsal root dilators.

Skin temperature experiments have been negative (Hinsey et al., 1934, 1938; Wybauw, 1938), a fact all the more important in view of the unanimity of opinion that the dorsal root dilators are almost exclusively distributed to the skin.

In view of the uncertainties, it seemed worthwhile to reinvestigate the experiments of Bayliss, especially since he himself emphasized the difficulty of demonstrating the phenomenon, and since he was unable to obtain completely satisfactory control observations. The following experiments were therefore designed to obtain records during vasomotor reflexes of the

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volume changes of a sympathectomized paw simultaneously with those of a completely denervated one.

METHOD. Cats or dogs anesthetized with dial (8 cats, 3 dogs) or urethane (8 cats, 3 dogs) were used. The lumbar sympathetic chains were removed from the third or fourth post-thoracic ganglion to the brim of the pelvis either on the day of the experiment or one to three weeks previously. Four of the cats were totally sympathectomized one to three months before the experiments. In order to minimize the fall of blood pressure produced by depressor stimulation, evisceration was performed in five of the cats and four of the dogs as recommended by Bayliss (1902). The adrenal glands were tied off in about one-third of the experiments. Denervation of a paw was effected by cutting the sciatic nerve at its emergence from the pelvis and the saphenous nerve in the femoral triangle.

In order to obtain reflex vasomotor effects, various nerves were stimulated. In the dogs, careful dissection of the vago-sympathetic trunk usually revealed a bundle of fibers, somewhat greyish pink in color, which had predominantly depressor effects (suggested by Dr. Stanley Nowak). In other experiments the left vagus and depressor, the nerve of Hering, or the median were employed. Stimulating current was supplied by a Harvard Inductorium with 3 or $4\frac{1}{2}$ volts in the primary circuit.

Since the dorsal root dilators have been found to be distributed primarily to skin areas, plethysmographs were designed to record changes in volume of the hind paws of the animals, as this region offers the greatest skin-muscle ratio of the limbs. Glass cylinders which just allowed the foot to enter were used and sealed around the foot with heavy vaseline well rubbed into the hair. In two acute experiments, performed on cats, muscle volume tracings were obtained from a conical metal plethysmograph, which was buried under the skin of the thigh. The chambers were connected by heavy rubber tubing to small segment capsules carrying a mirror at one side for optical recording. Calibration of each system was effected through a permanent side-arm connection with a straight 1 cc. pipette partially filled with mercury and fitted at the lower end with a closed rubber tube. Compression of the tube by a screw clamp, by raising the level of the mercury in the glass, had the effect of injecting a known volume of air into the plethysmograph system. This arrangement also provided a ready means of testing the recording system for air tightness. At the beginning of all experiments, and several times throughout their course, the plethysmographs were deflected to the limit of the recording scale by the introduction of air and observed for several minutes. Only if this test showed the plethysmograph seals to be tight were the records considered valid.

Since a rubber membrane served as the recording device, the measurements were not strictly volumetric, but none of the volume changes ob-

served involved a pressure change of more than 1.5 cm. of water. The slight theoretical disadvantage entailed was more than compensated for by the fact that the system was virtually inertialess when compared with volumetric recorders of comparable convenience. The sensitivity with a recording distance of 1 m. was such that 0.001 cc. change in volume equalled approximately 1 mm. on the record, and that a measurable change of paw volume showed readily with each heart beat. Active changes of caliber in the paw vessels could thus be estimated not only by modifications in the volume of the paw but also by changes in the pulse amplitudes. Passive changes of paw volume, especially those due to changes in venous pressure, gave little or no change in the pulse amplitude. Blood pressure was recorded optically from the carotid artery by a membrane manometer in the usual way.

Fig. 1

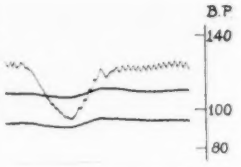

Fig. 2

Fig. 1. Negative effect in dog. Dial anesthesia, acute lumbar sympathectomy, evisceration, adrenals excluded, both vagi cut. Faradic stimulus during signal to left depressor nerve centrally, coil 7.5 cm. Upper tracing intact paw, lower tracing totally denervated paw, broad tracing blood pressure. Time in seconds in this and succeeding figures. *BP*: blood pressure.

Fig. 2. Negative effect in cat. Dial anesthesia, chronic bilateral lumbar sympathectomy (3 weeks' duration). Left vagus stimulated centrally, coil 10.5 cm. Reading downwards: blood pressure, sympathectomized paw, acutely denervated paw.

Results. The experiments failed to demonstrate any changes in paw volume which could be attributed to reflex stimulation of the dorsal root dilators. The usual result of stimulating either depressor or carotid sinus nerves in the animals with lumbar sympathectomy was a fall in blood pressure (slight in the eviscerated preparations) accompanied by a decline in volume of the paws. The shrinking of the totally denervated side was, within the limits of the method, comparable to that on the side with sympathectomy only (figs. 1 and 2). This was true in spite of the fact that in many of the experiments, of which figure 1 is typical, the fall of blood pressure on depressor stimulation was very small owing to the exclusion of the splanchnic area from the circulation. Passive effects were thus minimal as shown by the slight fall in the volume of the denervated paw. In contrast with these negative effects, it was easy to demonstrate the well-known reflex inhibition of the vasomotor tone in paws with intact

sympathetic supply, even in the face of much greater falls of blood pressure and shrinking of the denervated control paw.

Passive effects only were observed in the two experiments employing a muscle plethysmograph on an acutely sympathectomized thigh (cf. Jarisch, 1925).

An objection may be raised to the experiments in which the sympathetic chains were removed at the time of the experiment. It might be said that the vessels acutely deprived of their vasocontrictors would be maximally dilated and thus unable to respond to a reflex activation of the dorsal roots. It is well known (Bayliss, 1902), however, that such vessels do respond to direct stimulation of dorsal roots, a fact which was confirmed in the only two experiments of this sort attempted in this series (cats).

Rarely in the cats (3 out of 16) and more commonly in the dogs (4 out of 6) increases in the volume of the sympathectomized paw were encountered. At some stage in the experiment, the usual passive fall in paw volume con-

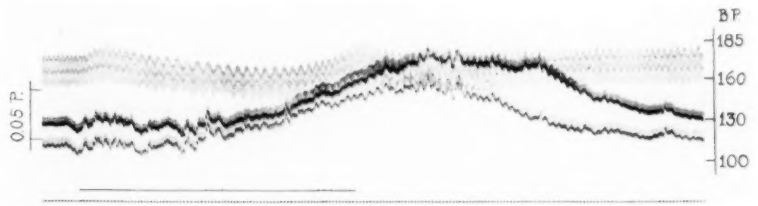

Fig. 3. False-positive effect in dog. Urethane anesthesia, acute lumbar sympathectomy, evisceration, both vagi cut. Depressor stimulation coil 10 cm. Reading downwards: blood pressure, sympathectomized paw, denervated paw. *P*: plethysmograph volume change in cubic centimeter.

sequent to depressor stimulation gave way to a rise in volume. This rise, however, generally took place in *both* the sympathectomized and the totally denervated paw. The increase in volume usually began about 20 to 30 seconds after the onset of stimulation, and continued until 10 to 20 seconds after stimulation ceased when a slow decline to the previous baseline set it. Figure 3 illustrates the most striking instance. The effect was inconstant, but in the dogs was repeatable to a greater or less degree at least three or four times during the experiment only to disappear without observable cause. The time relations suggest a humoral origin, but it occurred in several preparations in which the adrenals were ligated.

Changes in rate and depth of respiration brought about by depressor or carotid sinus stimulation could not be definitely correlated with the increases in paw volume, since the latter occurred during both increases and decreases in respiratory activity, and in one animal with bilateral pneumothorax and artificial respiration.

Discussion. Bayliss and others have pointed to the occasional increase

in volume of sympathectomized limbs seen during depressor stimulation as evidence of true reflex activation of dorsal root dilators. The experiments presented here show that when such changes take place, entirely similar changes may occur simultaneously in completely denervated areas, and therefore seriously impair the cogency of the existing plethysmographic evidence as applied to this problem. As was pointed out in the introduction to this paper, other direct methods of investigation have almost without exception yielded negative results. The only other serious support for true reflex activation of dorsal root dilators is to be found in the indirect evidence educed from the reflex changes of blood pressure in sympathectomized animals (Freeman and Rosenblueth, 1931; Rosenblueth and Cannon, 1934; Pinkston, Partington and Rosenblueth, 1936). This puzzling phenomenon may, however, find an explanation on other grounds, although attempts to do so have not so far been satisfactory (cf. Brown, 1940).

Various theoretical considerations may be mentioned which militate against the view that the dorsal root dilators participate in reflex adjustments of blood pressure by the circulatory proprioceptors. The dilator fibers in the dorsal roots have been shown to belong to the slowly conducting "C" group (Hinsey and Gasser, 1930). Anatomically (Ranson and Billingsley, 1916) and physiologically (Clark, Hughes and Gasser, 1935; Heinbecker and Bishop, 1935) this component of the dorsal roots has been shown to be concerned with pain sensation, and there are no grounds for believing that a specific portion of it is reserved for a vasomotor function. In fact the classical diagram of the axon reflex assumed the identity of the dorsal root dilators with sensory fibers. If this assumption is correct, it is hard to see how their central activation could take place without alteration of the normal synaptic activity involved in the transmission of pain.

The researches of Lewis (1927) and others have shown that the dorsal root dilators are principally concerned with localized reactions in response to damaging stimuli (i.e., inflammation). In accordance with this, responses evoked by direct stimulation have a relatively long latency and a long duration, up to fifteen minutes. Bayliss, observing this long duration, dismissed it as due to congestion from the pressure of the plethysmograph cuff, but later observations with other techniques have established the phenomenon (Langley, 1923; Wybauw, 1936; Lewis and Marvin, 1927).

The properties listed above would apparently render the dorsal root dilators unsuited for the purpose of making reflex adjustments of the blood pressure in response to the ever changing stimuli supplied by the receptors in the aortic arch and carotid sinus.

It is unfortunate, though unimportant for the present argument, that

we are unable definitely to state the origin of the dilator reactions observed in totally denervated paws. The caliber of blood vessels is of course influenced by many non-nervous factors one or more of which may account for the results obtained in this and other investigations. Whatever the nature of these effects, it seems clear that a dilatation observed in a sympathectomized hindlimb during a fall of blood pressure is not sufficient evidence that an extrasympathetic nervous control is in operation. We conclude that the theory that the vasodilator fibers in the dorsal roots can be activated reflexly from the central nervous system lacks any adequate direct support.

SUMMARY

Simultaneous recording of the volume of a sympathectomized and of a completely denervated paw during reflex depressor stimulation revealed no differences between the reactions of the blood vessels of the two sides, although both increases and decreases of paw volume were produced.

It is concluded that the existing evidence does not favor the concept of reflex activation of the dorsal root dilators.

REFERENCES

Bacq, Z. M., L. Brouha and C. Heymans. C. R. Soc. Biol. **112:** 208, 1933.
Bayliss, W. M. J. Physiol. **28:** 276, 1902.
Bishop, G. H., P. Heinbecker and J. L. O'Leary. This Journal **106:** 647, 1933.
Brown, G. L. and W. d'A. Maycock. J. Physiol. **97:** 273, 1940.
Clark, D., J. Hughes and H. S. Gasser. This Journal **114:** 69, 1935–36.
Corbin, K. B. and J. C. Hinsey. Proc. Soc. Exper. Biol. and Med. **32:** 368, 1934.
Derom, E. and K. S. Grimson. C. R. Soc. Biol. **131:** 1281, 1939.
Downman, C. B. B., A. E. Goggio, B. A. McSwiney and M. H. C. Young. J. Physiol. **96:** 14P, 1939.
Fofanow, L. L. and M. A. Tschalussow. Pflüger's Arch. **151:** 543, 1913.
Freeman, N. E. and A. Rosenblueth. This Journal **98:** 454, 1931.
Heinbecker, P. and G. H. Bishop. Res. Pbl. Assn. Res. N. and M. Dis. **15:** 226, 1935.
Hinsey, J. C. Annual Review Physiol. **1:** 407, 1939.
Hinsey, J. C. and H. S. Gasser. This Journal **92:** 679, 1930.
Hinsey, J. C. and R. A. Phillips. This Journal **123:** 101, 1938.
Jarisch, A. J. Physiol. **60:** 419, 1925.
Langley, J. N. J. Physiol. **58:** 49, 1923–24.
Lewis, T. The blood vessels of the human skin and their responses. Shaw, London, 1927.
Lewis, T. and H. M. Marvin. Heart **14:** 27, 1927.
Pinkston, J. O., P. F. Partington and A. Rosenblueth. This Journal **115:** 711, 1936.
Ranson, S. W. and P. R. Billingsley. This Journal **40:** 571, 1916.
Rosenblueth, A. and W. B. Cannon. This Journal **108:** 599, 1934.
Tournade, A. and J. Malméjac. C. R. Soc. Biol. **112:** 679, 1933.
Wybauw, L. C. R. Soc. Biol. **121:** 1377, 1936.
Arch. int. Physiol. **46:** 293, 1938.

THE ASCORBIC ACID, PHOSPHATASE AND CALCIUM CONTENT OF THE BLOOD OF GUINEA PIGS WITH VARYING DEGREES OF SCURVY[1]

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One of the characteristic symptoms of scurvy is the lack of intercellular material in the bones and the ease with which the bones of guinea pigs are fractured. Observations of this condition aroused our interest in the possible relation between ascorbic acid and calcium content of the blood and the phosphatase activity of the serum. This problem therefore has been investigated using groups of guinea pigs receiving a high ascorbic acid intake, a subnormal and a very low ascorbic acid intake.

METHODS. *Experimental animals.* Healthy guinea pigs, six to eight weeks of age, and weighing 280 to 300 grams were fed a vitamin C-free basal diet supplemented with different amounts of ascorbic acid. The basal diet consisted of rolled oats 36 per cent, bran 18 per cent, heated skim milk powder 30 per cent, dried yeast 5 per cent, hydrogenated fat (Crisco) 8 per cent, cod liver oil 2 per cent, sodium chloride 1 per cent. The experimental period lasted 34 days and at the end of that time the animals were destroyed and scored for scurvy by the method of Sherman, LaMer and Campbell (1922).

Determination of ascorbic acid. The ascorbic acid content of the oxalated blood plasma was determined by the micro-method of Farmer and Abt (1936) with an accuracy of ± 0.08 mgm. per 100 ml. of plasma.

Determination of serum calcium. Serum calcium was determined by the Tisdall (1923) modification of the Kramer and Tisdall (1921) method, but with the exception that the wash solution was added to the surface of the precipitate and not mixed with it. By this method 98 per cent of added calcium was recovered.

Determination of serum phosphatase. Phosphatase activity was determined by the King and Delory (1937) micro-method and is expressed

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[2] The data in this paper have been taken from a thesis submitted by W. Brewer in partial fulfillment of the requirement for the degree of master of science, 1939.

as units per 100 ml. of serum. Each unit is equal to the milligrams of phenol liberated from a di-sodium phenyl phosphate substrate in 15 minutes at 37°C. and pH 9.0. Color comparisons were made in a Duboscq colorimeter. The micro-method was chosen because of the small amount of serum required. According to King and Delory, the results obtained by this method are quantitatively equal to those obtained by the macro-method of King (1934). Brown (1936) in a comparative study of the different methods of phosphatase determination found that the method of King (1934) gave results consistent with those obtained by the Bodansky method (1932).

Blood samples. For the ascorbic acid determinations, blood was taken from the heart of the etherized animal using a syringe which had previously been rinsed with a 2 per cent solution of lithium oxalate. For the serum calcium and phosphatase determinations the throat of the animal was severed and the body drained of blood. Ether was reported by Bowman and Muntwyler (1937) to cause an increase in the amount of ascorbic acid excreted in the urine, and Ecker (1938) reported that there was a rise in serum ascorbic acid after ether anesthesia. Since ether might possibly have caused a change in the level of the blood constituents being studied a preliminary investigation was made with blood samples taken without ether and after 15 and 45 minutes under ether anesthesia. Plasma ascorbic acid and serum phosphatase were determined under these conditions, but insufficient blood was obtainable from each animal for similar studies on calcium content.

Plasma ascorbic acid values of blood samples taken from four animals without ether were 0.41, 0.30, 1.03 and 0.46 mgm. per 100 ml. whereas after seven minutes under ether anesthesia the plasma values were 0.44, 0.30, 0.97 and 0.44 mgm. per 100 ml. respectively. There was a rise in the plasma ascorbic acid content after 45 minutes of ether the respective values being 0.56, 0.36, 1.17 and 0.58 mgm. per 100 ml. Since there was no appreciable change in plasma ascorbic acid in seven minutes of ether, all blood values reported in this paper were taken within seven minutes of the time when ether was first given.

Determination of serum phosphatase activity of blood samples from three animals showed the following values: without ether, 9.2, 14.4, 12.0 units per 100 ml.; under ether for 15 minutes 9.5, 14.1, 11.5 units per 100 ml. and under ether for 45 minutes 12.3, 12.4 and 15.7 units per 100 ml. There was no change in phosphatase activity after 15 minutes of ether and therefore all blood samples for serum phosphatase determinations were collected within 15 minutes after administration of ether.

Blood samples for calcium determinations were taken at the same time as those for phosphatase.

Series I. Blood plasma ascorbic acid in relation to ascorbic acid intake.

The ascorbic acid content of the blood plasma of monkeys and man has been shown to be dependent upon the dietary intake of the vitamin (Farmer and Abt, 1935; Greenberg, Rinehart and Phatak 1937). Few data have been reported for blood plasma of guinea pigs and the available evidence does not indicate what is the ascorbic acid content of the blood of so-called normal animals or whether the ascorbic acid content is influenced by the diet. In order to clarify this question, blood determinations were made on all available guinea pigs in the laboratory; these included animals which had received 8 mgm. of crystalline ascorbic acid[3] daily for 34 days, and animals which had received 2 mgm. of ascorbic acid daily from lemon juice or from the crystalline form of the vitamin for the same period of time. Another group of animals being used for vitamin C assay of certain fruits received basal diet only for 13 days followed by daily fruit supplements which supplied approximately 0.5 mgm. of ascorbic acid daily for 21 days. A group of negative controls was maintained on the vitamin-C-free basal diet until they showed signs of severe scurvy; blood samples were then taken and the animals were destroyed.

The mean plasma ascorbic acid values for the different groups of animals are summarized in table 1. The negative controls showed no measurable amount of ascorbic acid in the blood plasma. Those animals which had been made scorbutic and then fed various fruit supplements supplying approximately 0.5 mgm. ascorbic acid daily had scurvy scores ranging from 0 to 11 and the blood plasma ascorbic acid content was 0.16 mgm. per 100 ml. with a standard deviation of ± 0.07. The mean value for plasma ascorbic acid of animals receiving 2.0 mgm. of ascorbic acid daily was 0.22 mgm. per 100 ml. with a standard deviation of ± 0.03. Statistical treatment of the data of these two groups as described by Snedecor (1938) for determination of t gives a t value of 4.962 which shows that the difference between the two groups is significant.

Guinea pigs which received 8 mgm. of ascorbic acid daily had a mean plasma content of 0.54 mgm. per 100 ml. with a standard deviation of ± 0.11; the t value of 9.119 shows this to be a highly significant increase in blood ascorbic acid over the group receiving 2.0 mgm. daily. Thus the ascorbic acid content of the blood plasma of guinea pigs is seen to be dependent on the intake.

Series II. Ascorbic acid, calcium, and phosphatase content of blood of guinea pigs with varying degrees of scurvy. In this series three groups of animals were used and were comparable in every way except for the amount of vitamin C fed and the consequent degree of scurvy. In group I there were 8 animals which had received 8 mgm. ascorbic acid daily throughout

[3] We are indebted to Merck and Company, Rahway, New Jersey for a generous supply of ascorbic acid.

the experimental period; these animals appeared to be healthy and increased rapidly in weight during the period. There were 21 animals in group II and on autopsy all showed mild scurvy with scores ranging from one to eight. In group III there were 10 animals all of which showed severe scurvy with scores of 9 to 16. The ascorbic acid content of the blood plasma, and the phosphatase and calcium content of the serum of each animal were determined with the exception of six animals where the blood supply was inadequate for the calcium determination. The data are summarized in table 2. No measurable amount of ascorbic acid was found in the blood plasma of animals with severe scurvy; those with mild scurvy had an average of 0.20 mgm. ± 0.062[4] per 100 ml. and the normal animals averaged 0.57 mgm. ± 0.105 per 100 ml. The difference between the two latter groups is significant, t value being 9.38.

TABLE 1

Ascorbic acid content of blood plasma of guinea pigs with different intakes of ascorbic acid

ASCORBIC ACID INTAKE DAILY	NUMBER OF ANIMALS		SCURVY SCORE	ASCORBIC ACID PER 100 ML. PLASMA	STANDARD DEVIATION	t VALUE
	Males	Females				
				mgm.		
8 mgm. for 34 days	6	4	0	0.54	0.11	9.119
2 mgm. for 34 days	6	6	0	0.22	0.03	
Basal diet only for 13 days then 0.5 mgm. for 21 days	40	33	0–11	0.16	0.07	4.962

In the normal animals the mean phosphatase value in units per 100 ml. of serum was 17.0. Mild scurvy caused a slight rise in phosphatase activity to 21.0 units and the t value for the mean difference between these two groups was 2.29 which is barely significant. The animals with severe scurvy showed a marked lowering in phosphatase to 6.8 units. The t value of the mean difference in phosphatase value between this group of animals with severe scurvy and the normal animals was 5.49; the t test also showed that the difference between the means for the group with mild scurvy and the group with severe scurvy was significant, the value being 9.47.

The mean serum calcium content was approximately the same for all three groups being 13.1, 13.9 and 13.0 mgm. per 100 ml. of serum.

A comparison was also made of the blood constituents for each sex but in some groups insufficient numbers of animals had been used to draw any conclusions. There appeared to be no difference in the ascorbic acid

[4] Standard deviation.

TABLE 2

Ascorbic acid, phosphatase and calcium content of blood of scorbutic and non-scorbutic guinea pigs

DEGREE OF SCURVY	ANIMAL NO.	SEX	SCURVY SCORE	PLASMA ASCORBIC ACID	SERUM PHOSPHATASE	SERUM CALCIUM
				mgm. per 100 ml.	*units per 100 ml.*	*mgm. per 100 ml.*
None (8 mgm. ascorbic acid daily)	1	M	0	0.69	24.9	12.6
	2	M	0	0.72	20.2	11.5
	3	M	0	0.57	15.2	15.3
	4	F	0	0.55	17.6	14.1
	5	M	0	0.53	13.8	14.6
	6	M	0	0.62	13.6	14.4
	7	M	0	0.44	17.8	11.7
	8	F	0	0.44	12.6	10.8
Mean				0.57	17.0	13.1
Mild (score 1–8)	9	F	2	0.21	22.4	
	10	F	5	0.17	20.5	9.8
	11	M	1	0.21	17.8	15.1
	12	M	3	0.23	21.6	14.9
	13	M	3	0.16	17.4	13.7
	14	F	5	0.16	17.0	13.3
	15	M	1	0.34	16.7	13.4
	16	M	1	0.26	15.8	12.6
	17	M	1	0.21	25.6	12.4
	18	M	3	0.30	29.8	12.6
	19	M	1	0.21	24.0	15.0
	20	M	3	0.22	27.4	14.0
	21	F	1	0.23	16.3	10.2
	22	F	3	0.10	16.8	11.4
	23	M	6	0.10	23.6	11.9
	24	F	4	0.17	17.1	12.9
	25	M	4	0.25	26.1	12.3
	26	F	2	0.24	20.2	13.8
	27	F	4	0.26	14.8	
	28	M	7	0.12	27.0	15.2
	29	F	1	0.15	24.1	
Mean				0.20	21.0	13.0
Severe (score 9–16)	30	F	9	0	11.6	15.3
	31	F	11	0	11.4	14.7
	32	F	9	0	8.3	
	33	F	12	0	4.6	11.9
	34	F	14	0	3.4	12.4
	35	F	13	0	5.2	
	36	M	15	0	4.2	14.5
	37	M	10	0	6.6	15.5
	38	M	14	0	9.5	
	39	M	15	0	2.7	13.0
Mean				0	6.8	13.9

content of the blood plasma of males and females. In the group receiving 2 mgm. of ascorbic acid per 100 mgm. plasma the average for six males was 0.24 mgm. and for six females was 0.21 mgm. In the group receiving 0.5 mgm. of ascorbic acid the average plasma content for 40 males was 0.15 mgm. and for 33 females it was 0.17 mgm.

Similarly the calcium content of the blood of the two sexes was comparable. In the group with scurvy scores ranging from 1 to 8 the average calcium value for 12 males was 13.6 mgm. per 100 ml. and for 6 females was 11.9 mgm. In the group with scurvy scores of 9 to 16 the average for three males was 14.3 mgm. and for four females 13.6 mgm. per 100 ml.

There was considerable variation in the phosphatase values within each group; for those having scurvy scores of 1 to 8, a group of 9 females had an average of 18.8 phosphatase units per 100 ml. and a group of 12 males 22.7 units.

Series III. Ascorbic acid, calcium, and phosphatase content of blood of animals with restricted food intake. Guinea pigs suffering from scurvy show loss of appetite and lowered food intake, and it is possible that the lowered food consumption of the scorbutic animals studied in series I and II might have been an influencing factor on the levels of the blood constituents. In this series, therefore, the paired-feeding method was used so that the food intake for each pair of animals was comparable. Six pairs of guinea pigs were used, one animal of each pair received basal diet only and the other received 8 mgm. of ascorbic acid daily in addition to the amount of basal diet equivalent to that which had been consumed by the corresponding pair-mate on the previous day. The experimental period was 34 days except for two animals on basal diet only that lived 26 and 28 days and the pair-mates were killed on the corresponding day.

The ascorbic acid, calcium, and phosphatase values are summarized in table 3. The scorbutic animals had scurvy scores ranging from 9 to 15 and no measurable amount of ascorbic acid in the plasma. Pair-mates receiving 8 mgm. of ascorbic acid daily had plasma values ranging from 0.31 mgm. to 0.41 mgm. per 100 ml. which is considerably lower than the ascorbic acid content of the blood plasma of the animals in series II with the same ascorbic acid intake but unrestricted food consumption.

The serum phosphatase activity of the animals receiving 8 mgm. of ascorbic acid daily was markedly greater than that of the scorbutic pair-mates but was lower than the mean value for animals in series II that also received 8 mgm. ascorbic acid daily and unrestricted food intake.

The serum calcium levels for the pair-mates in this series were comparable and were similar to the animals in series II.

In table 3 the weight gains for the pairmates are also shown. Although the average daily food intake was approximately the same for each pair

of animals those receiving ascorbic acid daily showed much greater gains in weight. McHenry, Reedman and Sheppard (1938) have reported similar findings and attribute the weight difference to the diminished retention of water in the bodies of animals which lack ascorbic acid (Sheppard, McHenry 1939).

Discussion. The data reported here clearly demonstrate that the ascorbic acid content of the blood of guinea pigs is dependent on the dietary intake of this substance and is not caused by the lowered food intake which accompanies the development of severe scurvy. These findings are in

TABLE 3

Ascorbic acid, calcium and phosphatase content of blood of guinea pigs in paired feeding study

GUINEA PIG NUMBER	SEX	ASCORBIC ACID INTAKE PER DAY	BODY WEIGHT				AVERAGE DAILY FOOD INTAKE	EXPERIMENTAL PERIOD	SCURVY SCORE	PLASMA A.-A.	SERUM PHOSPHATASE	SERUM CALCIUM
			Initial	Maximum	Final	Total gain						
		mgm.	*grams*	*grams*	*grams*	*grams*	*grams*	*days*		*mgm. per 100 ml.*	*units per 100 ml.*	*mgm. per 100 ml.*
1	M	0.0	300	415	319	19	15	34	13	0	5.2	
2	M	8.0	291	390	360	69	15	34	0	0.40	15.8	13.0
3	F	0.0	308	330	235	−73	12	28	14	0	3.4	12.4
4	F	8.0	302	316	290	−12	10	28	0	0.32	5.0	13.4
5	F	0.0	312	316	215	−97	11	26	12	0	4.6	11.9
6	F	8.0	305	331	312	7	11	26	0	0.41	11.7	14.8
7	M	0.0	304	435	367	63	20	34	9	0	6.6	15.5
8	M	8.0	313	470	440	127	17	34	0	0.40	15.2	12.8
9	M	0.0	302	302	197	−105	13	34	15	0	2.7	13.0
10	M	8.0	305	385	360	55	13	34	0	0.34	6.6	15.0
11	F	0.0	300	300	177	−123	10	34	14	0	9.5	
12	F	8.0	296	335	272	−24	10	34	0	0.31	11.4	13.8

agreement with those of other workers who have reported on the blood ascorbic acid of man and of monkeys in relation to the diet. Ecker and co-workers (1938) have reported values of 1.04 to 2.13 mgm. per 100 ml. of blood serum for guinea pigs fed green food for eight days which is considerably higher than the values obtained in this study for animals fed 8 mgm. ascorbic acid daily.

Serum phosphatase activity has been reported to be affected by abnormal conditions such as jaundice and various bone diseases as well as scurvy. Smith and Maizels (1932) have reported that there was a definite

increase in serum phosphatase activity in rickets in young children and this serum phosphatase rise has been recommended as a valuable index of early cases of rickets (Barnes and Carpenter, 1937). The bone changes taking place in scurvy are apparently caused by a lack of formation of intercellular substances (Wolbach, 1926) and a cessation of ossification and growth. This may account for the low phosphatase values reported in cases of scurvy in children (Smith and Maizels, 1932). Scoz and co-workers (1937) found first an increase in phosphatase activity and then a decrease during the progress of scurvy in guinea pigs. The data reported here show that animals with mild scurvy as indicated by scurvy scores of one to eight, showed some increase in serum phosphatase activity but statistical analysis shows that the increase is probably not significant. However, in animals with severe scurvy the serum phosphatase activity was significantly lower than that of healthy animals. Paired feeding experiments demonstrated that the degree of scurvy and not the restricted food intake was the cause of lowered phosphatase activity of the serum since the pair mates with similar food intake but adequate ascorbic acid had markedly higher phosphatase values.

Our findings with regard to the calcium content of the blood serum are in agreement with those of Goettsch and Key (1928) and Humphrey and Zilva (1931) who found a wide range in the serum calcium but no indication that these changes paralleled scurvy. Lowered food intake in the later stages of scurvy had no effect on the calcium level of the blood.

SUMMARY

The ascorbic acid, calcium, and phosphatase of the blood of guinea pigs receiving varying amounts of ascorbic acid as supplements to the basal ration were studied. The ascorbic acid content of the blood was found to be negligible in scurvy and to increase as the dietary intake was increased. A daily supplement of 8 mgm. ascorbic acid during the experimental period gave a mean blood value of 0.54 mgm. per 100 ml. of plasma; the corresponding value was 0.22 mgm. per 100 ml. when the daily intake was 2 mgm. ascorbic acid.

The phosphatase activity of the serum was increased slightly in mild scurvy and decreased markedly in cases of severe scurvy.

Serum calcium values remained unchanged in scorbutic animals.

Differences observed in phosphatase values and ascorbic acid content of the blood were not attributable to sex or to the amount of basal diet eaten, and were unchanged during 15 minutes of ether anesthesia.

REFERENCES

BARNES, D. J. AND M. D. CARPENTER. J. Pediat. **10:** 596, 1937.
BODANSKY, A. J. Biol. Chem. **99:** 197, 1932.

BOWMAN, D. E. AND E. MUNTWYLER. Proc. Soc. Exper. Biol. and Med. **35:** 557, 1937.
BROWN, P. C. Am. J. Med. Tech. **2:** 1, 1936.
ECKER, E. E., L. PILLEMER AND D. WERTHEIMER. J. Immunol. **34:** 39, 1938.
FARMER, C. J. AND A. F. ABT. Proc. Soc. Exper. Biol. and Med. **32:** 1625, 1935. Proc. Soc. Exper. Biol. and Med. **34:** 146, 1936.
GOETTSCH, M. AND K. M. KEY. Quart. J. Pharm. **1:** 168, 1928.
GREENBERG, L. C., J. F. RINEHART AND N. M. PHATAK. Proc. Soc. Exper. Biol. and Med. **35:** 135, 1937.
HUMPHREY, F. AND S. S. ZILVA. Biochem. J. **25:** 579, 1931.
KING, E. J. AND A. R. ARMSTRONG. Canad. M. A. J. **31:** 376, 1934.
KING, E. J., G. A. D. HASLEWOOD AND G. E. DELORY. Lancet **232:** 886, 1937.
KRAMER, B. AND F. F. TISDALL. J. Biol. Chem. **47:** 475, 1921.
MCHENRY, E. W., E. J. REEDMAN AND M. SHEPPARD. Biochem. J. **32:** 1302, 1938.
SCOZ, G., C. CATTANEO AND M. C. GABRIELLI. Enzymologia **3:** 29, 1937.
SHEPPARD, M. AND E. W. MCHENRY. Biochem. J. **33:** 655, 1939.
SHERMAN, H. C., V. LAMER AND H. L. CAMPBELL. J. Am. Chem. Soc. **44:** 165, 1922.
SMITH, J. AND M. MAIZELS. Arch. Dis. Child. **7:** 149, 1932.
SNEDECOR, G. W. Statistical methods. Collegiate Press, Inc., Ames, 1938.
TISDALL, F. F. J. Biol. Chem. **56:** 439, 1923.
WOLBACH, S. B. AND P. R. HOWE. Arch. Path. and Lab. Med. **1:** 1, 1926.

A HISTAMINE-LIKE SUBSTANCE IN THE POST-PARTUM RABBIT UTERUS[1]

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Among the characteristic and consistent changes in the rabbit uterus during the final stages of pregnancy, three may be especially important in the sequence of events leading to parturition. Two to three days before delivery the following changes can be observed: 1, increased color, of vascular origin, approaching a bluish-red on the day of delivery (Markee and French, 1934); 2, increased volume of the entire uterus reaching a maximum on the day of delivery, then slowly receding for 6 to 8 days post-partum (Markee and Andersen, 1934), and 3, increased amounts of intercellular fluid, or edema, reaching a peak on the day of delivery, then gradually receding for 6 to 8 days after delivery (Krichesky, unpublished).

In attempting to determine whether a full term or immediately post-partum, edematous uterus contains a local factor capable of inducing edema in a non-pregnant uterus, it was found (Krichesky and Pollock, 1940) that a crude saline extract of rabbit uterus, taken within a few hours after delivery, is extremely toxic to non-pregnant animals when injected intravenously in relatively small doses. This study was undertaken to confirm these previous findings and to determine, if possible, the nature of the toxic substance in extracts of full term edematous uteri.

MATERIALS AND METHODS. A total of 71 rabbits were used in this study; of these 54 were non-pregnant females, 12 were females in late stages of pregnancy or a few days post-partum, and 5 were males.

Four experiments were carried out: 1, to determine the toxicity of saline extracts of post-partum rabbit uteri; 2, to ascertain the toxicity of non-pregnant uteri and of skeletal muscle from post-partum rabbits; 3, to establish whether or not blood serum or whole blood from animals in late stages of pregnancy and immediately post-partum is toxic, and 4, to arrive at the nature of the toxic factor in post-partum uterine extracts.

In the first experiment all extracts were prepared as follows: Uteri were excised within a few hours after delivery, weighed, and ground to a mash with washed sand. A volume of saline equal to half the weight in grams of the fresh tissue was added. The mixture was shaken intermittently for

[1] Aided by a research grant from the University of California.

30 minutes, then centrifuged. The supernatant fluid was collected and the residue re-extracted. The supernatant fluids from the two extractions were combined and saline added until 1 cc. of the final solution represented 0.5 gram of fresh tissue. Merthiolate (1:50,000) was added and the extracts stored in a refrigerator. All extracts were used within four days of their preparation.

In the second experiment extracts of non-pregnant uteri and of muscle were prepared in the manner described. Abdominal muscles and portions of the thigh muscles were taken from post-partum animals used in experiment 1.

For experiment, blood was collected from the marginal ear vein of donor rabbits. Serum alone was used in some instances and whole blood in others.

It soon became evident that the toxic factor in post-partum uterine extracts was histamine-like in its action. For this reason, in the fourth experiment attempts were made to inactivate the toxic substance by using the enzyme histaminase (Torantil 360).[2] Histaminase in various proportions was added to both histamine and to toxic uterine extracts, the pH adjusted to 7.2 and the mixtures incubated for 24 hours at 37.5°C., then filtered, and the filtrate injected.

All animals were injected intravenously with a maximum single dose of 10 cc. Where larger doses were given they were administered by an additional maximum of 10 cc. on each successive day.

RESULTS. *Experiment 1. Toxicity of post-partum uterine extracts.* A total of 9 extracts were prepared from rabbit uteri taken between 2 and 12 hours after delivery, 10 within 7 hours and only two as long as 10 and 12 hours post-partum. These were injected intravenously into a total of 23 non-pregnant animals. The average lethal dose was 5.6 cc. representing 2.8 grams of fresh tissue. The potency of the individual extracts varied considerably, but in all instances the recipients succumbed within two minutes after injection. The least toxic extract required an average dose of 14.5 cc. to cause death. In the preparation of this extract the excised uterus was allowed to stand in the refrigerator for approximately 34 hours before extraction and it is possible that the toxic factor was partially destroyed or inactivated during that time. Only one extract was lethal in doses as small as 0.5 cc.

Young and old animals of both sexes proved equally susceptible. Although complete data were not obtained it appeared that the lethal dose was approximately proportional to body weight.

In addition to the animals mentioned above, three rabbits were injected

[2] Histaminase (Torantil 360) was generously supplied by the Department of Medical Research, Winthrop Chemical Company, Inc.

intraperitoneally. When given by this route the extracts also proved fatal, although a larger dose was required and a longer time elapsed before death.

Since there are large necrotic areas and masses of autolyzed cells in the post-partum uterus, the possibility existed that animals possessing such a uterus might be less susceptible to toxic extracts than non-pregnant animals. Toxic uterine extracts were injected into the following animals: one animal one day pre-partum, two animals on the day of delivery and two animals one day post-partum. These five animals died within two minutes of administration of approximately the same quantity producing death in non-pregnant animals. Thus the presence of an edematous uterus *in situ*, containing the toxic factor, does not influence the susceptibility of the animal to extracts of known toxicity.

Experiment 2. Effects of extracts of non-pregnant uterus and of muscle. In this experiment extracts of non-pregnant uteri and of muscles were prepared in the manner described for post-partum uteri, each cubic centimeter representing 0.5 gram fresh tissue.

Five extracts of non-pregnant uteri were injected intravenously into a total of 8 animals. The average dose was 26.2 cc. representing 13.1 grams of fresh tissue. Four of these extracts proved harmless in doses ranging from 15 to 37.5 cc., while one extract proved toxic in a dose of 20 cc. All animals received large doses and even in the only case of toxicity in an extract of non-pregnant uterus the lethal dose was almost five times greater than the average dose employed in experiment 1. These results indicate that the toxic factor present in post-partum uterine extracts is either absent or present in only low concentrations in non-pregnant uteri.

As a further control for experiment 1, four extracts were prepared from skeletal muscle taken from the same animals providing uterine material for extracts used in experiment 1. Six non-pregnant rabbits were given an average dose of 41.7 cc. of this material, representing 20.8 grams of fresh tissue. In no case did the muscle extracts prove fatal, indicating that if the toxic factor was present its concentration was too low to produce symptoms in the quantities used.

Experiment 3. Effects of blood serum and of whole blood from gravid and post-partum rabbits on non-pregnant animals. During late stages of pregnancy and immediately post-partum the rabbit uterus contains a larger volume of blood than does the non-pregnant uterus or muscle, consequently the possibility existed that the toxic factor in post-partum uterine extracts was present in the blood rather than in the tissues. Blood was therefore collected from the marginal ear vein of both gravid and post-partum animals. Blood serum alone was injected into 5 rabbits in quantities as high as 20 cc. Whole blood was given to two animals in doses of 16 cc. All animals survived this treatment, indicating that neither blood serum

nor whole blood, in the amount used, contained a sufficiently high concentration of the toxic factor to be lethal.

Experiment 4. Inactivation of histamine solutions and toxic uterine extracts by incubation with histaminase. All animals that succumbed from an injection of toxic uterine extracts exhibited symptoms preceding death strikingly similar to those described for fatal anaphylaxis. There was a blanching of the eye and ear, extreme respiratory difficulty, accompanied by jerking motions of the head and body. On autopsy the lungs were characteristically distended and the right chambers of the heart engorged. The phenomenon was not considered an anaphylaxis, however, since none of the animals had been previously sensitized.

Since several investigators reported a possible relation between histamine and anaphylaxis (Dale and Laidlaw, 1910; Bally, 1929; Dragstedt and Gebauer-Fuelnegg, 1932; Code, 1939 and others), it seemed possible that the toxic factor in post-partum uterine extracts might be histamine or histamine-like.

Four rabbits were injected with a histamine solution containing 1.0 mgm. per cubic centimeter. Two of these were given a total of 0.5 cc., or 0.5 mgm., and two a total of 1.0 cc., or 1.0 mgm. The smaller dose produced severe shock from which both animals recovered, while the larger dose killed the animals in less than two minutes and reproduced accurately the symptoms and pathological changes following administration of toxic uterine extracts. This provided further evidence that the toxic factor might be histamine or a histamine-like substance.

Best and McHenry (1930, 1931) reported the isolation of an enzyme, histaminase, which destroyed the activity of histamine when incubated with it. The enzyme itself is not toxic even when given in relatively high concentrations (Barlow, unpublished). Three rabbits were given 3, 5 and 10 units respectively of histaminase intravenously and exhibited no ill effects. In accord with the work of Best and McHenry (1930, 1931) two rabbits given 4 and 5 times the lethal dose of histamine previously incubated with histaminase also exhibited no symptoms, indicating that the amine had been inactivated.

Since it appeared that the toxic factor in uterine extracts might be histamine, extracts of known toxicity were incubated with histaminase, then tested for activity. To 40 cc. of one extract 25 units of histaminase were added, the pH adjusted and the mixture incubated for 24 hours at 37.5°C. and then filtered. The filtrate was given to 4 animals in doses of 9 cc. (9 times the lethal dose of this extract before inactivation). None of these animals succumbed and only one exhibited symptoms of shock. In another instance, 50 cc. of an extract were incubated with 20 units of histaminase and the filtrate given to four animals in doses of 12 cc. Although this was four times the lethal dose of this extract before treatment

with histaminase, it failed to produce any of the characteristic symptoms of toxicity.

The addition of 8 units of histaminase to 12.0 cc. of another extract but without subsequent incubation, had no effect on toxicity when tested on two animals. The incubation of an extract of known toxicity but without added histaminase also failed to alter the toxicity of the material. These results indicate that the toxic factor in extracts of the post-partum rabbit uterus is inactivated by the action of histaminase and consequently it may be assumed that the toxic factor is histamine or a histamine-like substance.

Discussion. It is unusual that saline extracts of normal tissue are toxic to animals of the same species. However, such extracts of the post-partum rabbit uterus, taken within 12 hours after delivery, are extremely so. Furthermore, the absence of toxicity from similar extracts of the non-pregnant uterus indicates that the toxic material is associated with a particular physiological state.

Our results indicate that the toxic factor is either histamine or a substance histamine-like in its physiological properties and chemical structure. Reference has been made to the reported relation between histamine and anaphylaxis. Symptoms preceding death and pathological changes after death, following administration of toxic uterine extracts, are accurately reproduced by lethal quantities of histamine.

A more specific test is the inactivation of the toxic material by incubation with histaminase. By this means the activity is destroyed and some structural similarity to histamine is inferred, since it is suggested by Best and McHenry (1930) that histaminase acts on histamine by destruction of the imidazole ring.[3]

The presence of large amounts of a substance with histamine-like action may possibly explain some of the characteristic histological and physiological changes in the full term rabbit uterus. The intensity of color, of vascular origin, increases to a bluish-red on the day of delivery and then slowly diminishes (Markee and French, 1934). Observations by one of us (Krichesky, unpublished) indicate that the capillary bed and small venules of the endometrium are greatly dilated and filled with blood cells beginning two days pre-partum until two days post-partum. This can be explained by a constriction of the veins with a consequent dilatation in venules and capillaries, producing stasis. Capillary dilatation and venous constriction after histamine has been reported by several investigators (Feldberg, 1927;

[3] In addition, extraction of histamine by the method of Barsoum and Gaddum, (1935) was carried out on one extract and the resulting substance, even in dilute solutions, produced contractions of the guinea pig ileum immersed in Tyrode's solution, providing further evidence that the toxic factor is histamine or histamine-like in its properties.

Ruhman, 1932; Sturm and Dauter, 1937 and others). By this action on the vascular system, it is possible that a substance with histamine-like properties may account for the increased intensity of color in the uterus and endometrium toward the end of pregnancy.

The presence of a substance with histamine-like activity may explain adequately both the growth in size of uterus and endometrium during the last two or three days of pregnancy (Markee and Andersen, 1934), and the accumulation of intercellular fluid (Krichesky, unpublished). The H-substance postulated by Lewis (1927) in the triple response of the skin to histamine or mechanical stimulation, suggests increased capillary permeability. It has been demonstrated also by Ravenna (1930), Kiuchi (1937), and others, that capillary permeability is increased by the administration of histamine. Consequently, the presence of large amounts of histamine, or a substance with histamine-like activity, because of its effect on capillary walls, may produce an edema resulting in an increase in the size of the organ.

The expulsion of the embryos at the end of pregnancy may be influenced also by the presence of a high concentration of a histamine-like substance in the full term rabbit uterus. Dale and Laidlaw (1910) recorded the contractions of the uterus in a pithed cat after administration of 1.0 mgm. histamine intravenously. *In vitro* experiments by tum Suden (1934) using rats, and Sugimoto (1937), using several different animals, obtained uterine contractions on addition of histamine. In view of the work of Barsoum and Gaddum (1935) who found that rabbit blood contains the highest histamine equivalent in the series of common laboratory animals studied, it is not surprising that Sugimoto found the rabbit uterus least sensitive in the series of animals he studied.

Bourne and Burn (1927), however, in some clinical studies, reported that although injections of 2 mgm. of histamine under the skin produced powerful but short lived contractions of the uterus, labor was not accelerated. They do not consider histamine a factor initiating termination of pregnancy because, in their experiments, uterine muscle activity ceased after the effect of histamine had worn off.

Little is known quantitatively or qualitatively of histamine in the full term human uterus, but in the rabbit, at least, it is difficult to conceive of the presence of large amounts of a histamine-like substance capable of exerting such a powerful effect on smooth muscles without its taking some part in the contractions of the uterus leading to parturition. In agreement with this view is the work of Danforth (1939) who reported that a high histaminase content of the human placenta is accompanied by weak uterine contractions whereas a low histaminase content of the placenta is accompanied by vigorous contractions. This suggests that in the case of high histaminase content, histamine or histamine-like substances are inactivated

resulting in weak uterine contractions, strong contractions being present only when the histaminase content is low.

In addition, Marcou and Atanasia-Vergu (1937) find that the concentration of blood histamine is lower in pregnant than in normal women and that the concentration falls very low, less than 0.05 gamma per cubic centimeter, during labor. They suggest that the histamine is taken up by other organs, but it is possible, in the rabbit at least, that it is taken up by the uterus itself, since a high concentration of a histamine-like substance is present in the rabbit uterus at that time. The mechanism of this decrease is unknown and Marcou and Atanasia-Vergu postulate that a low level of blood histamine, normally a vasodilator, may reduce post-partum hemorrhage. However, high concentrations of histamine are known to produce constriction of the arteries (Feldberg, 1927; Wilson, 1936 and Kiuchi, 1937). The possibility exists, therefore, that post-partum hemorrhage may be reduced not because of a low concentration of histamine in the blood but because of arterial constriction due to a high concentration of histamine or a histamine-like substance in the uterus at that time.

SUMMARY

1. Saline extracts of post-partum rabbit uteri, taken within 12 hours after delivery, contain a toxic substance lethal to non-pregnant animals.

2. With one exception, the toxic factor was not present in non-pregnant uteri or in skeletal muscle, blood serum, or whole blood of rabbits in late stages of pregnancy or one day post-partum.

3. Animals one day pre-partum to one day post-partum are no less susceptible to the toxic factor than are non-pregnant animals.

4. The toxic factor is probably histamine or a histamine-like substance. It resembles histamine in physiological properties and is readily inactivated by incubation with histaminase.

5. The presence of histamine or a histamine-like material in the full term and post-partum rabbit uterus is discussed in relation to events leading to parturition, i.e., color change, edema, muscle contractions and post-partum hemorrhage.

REFERENCES

BALLY, L. H. J. Immunol. **17:** 191, 207, 223, 1929.
BARSOUM, G. S. AND J. H. GADDUM. J. Physiol. **85:** 1, 1935.
BEST, C. H. AND E. A. MCHENRY. J. Physiol. **70:** 349, 1930.
Physiol. Rev. **11:** 371, 1931.
BOURNE, A. AND J. H. BURN. Pharmacol. J. **119:** 485, 1927.
CODE, C. F. This Journal **127:** 78, 1939.
DALE, H. H. AND P. P. LAIDLAW. J. Physiol. **41:** 318, 1910.
DANFORTH, D. N. Proc. Soc. Exper. Biol. and Med. **40:** 319, 1939.
DRAGSTEDT, C. A. AND E. GEBAUER-FUELNEGG. This Journal **102:** 505, 1932.
FELDBERG, W. J. Physiol. **63:** 211, 1927.

KIUCHI, S. Sei-i-kai Med. J. **55:** 1354, 1937.
KRICHESKY, B. AND W. POLLOCK. Science, **91:** 410, 1940.
LEWIS, T. Shaw, London, 1927.
MARCOU, I. AND E. ATANASIU-VERGU. Bull. acad. med. Roumanie **3:** 359, 1937.
MARKEE, J. E. AND E. ANDERSEN. Anat. Rec. **58:** Suppl. 78, 1934.
MARKEE, J. E. AND H. M. FRENCH. Anat. Rec. **58:** Suppl. 79, 1934.
RAVENNA, P. Sperimentale **84:** 165, 1930.
RUHMAN, W. Ztschr. ges. Exper. Med. **85:** 563, 1932.
STURM, A. AND H. DAUTER. Arch. Exper. Path. u. Pharmakol. **185:** 368, 1937.
SUGIMOTO, T. Proc. Jap. Pharmacol. Soc. **11:** 121, 1937.
TUM SUDEN, C. This Journal **108:** 416, 1934.
WILSON, H. C. J. Pharmacol. **56:** 97, 1936.

THE IN VITRO SECRETION OF ACID BY THE GASTRIC MUCOSA OF THE FROG

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In 1930 Delrue (1) reported that the isolated gastric mucosa of the frog, when mounted properly in a dual-chambered bath, will secrete acid and will respond to the addition of drugs. The same author subsequently demonstrated (2) the effects of varying the ionic composition of the artificial medium on the *in vitro* production of acid. The theoretical importance of this approach to the study of the mechanism of acid formation by the gastric glands is apparent. However, to our knowledge, this work has never been confirmed, nor has the method been applied to the problem of acid formation.

METHODS. From a pithed frog the stomach, together with a short segment of the esophagus and duodenum, was removed and washed out with isotonic saline. An incision was made along the lesser curvature through the muscularis layer and the intact mucosal layer was dissected free. The resulting tube of mucosa was cut along the lesser curvature and spread out as a flat membrane over a hole, one centimeter in diameter, made in the center of a thin Monel metal plate 4 cm. square. Another similar metal plate was placed on top of the mucosa and the two were bolted together with the mucosa held firmly between them. This assembly was then mounted between two small glass chambers shaped like $CaCl_2$ tubes. Each chamber had a hole $\frac{1}{2}$ inch in diameter ground on one side. The chambers were clamped together with a modified burette clamp in such a way that the edges of the holes in the glass chambers were pressed in water-tight contact with rubber gaskets which had been cemented around the holes in the metal plates. The contents of the two chambers were thus separated only by the thin layer of the gastric mucosa.

One chamber with a capacity of 6 cc. was filled with 0.7 per cent NaCl solution through which air was bubbled; this solution bathed the natural secretory surface of the mucosa. The other chamber with a capacity of 15 cc. was filled with an artificial nutrient solution aerated with 95 per cent oxygen and 5 per cent carbon dioxide; this solution bathed the side of the mucosa which had originally been in contact with the muscularis.

The fluid level in both chambers extended above the upper edge of the mucosa, but higher on the nutrient than on the secretory side. This caused the loose membrane to balloon slightly into the secretory solution, so that the agitation produced by aeration effectively stirred the solution and prevented a tenacious layer of mucus from forming on the mucosal surface.

The outlet at the bottom of both chambers permitted convenient emptying, and, in the case of the secretory chamber, it provided a connection for a salt bridge for the measurement of pH. The bridge was filled with

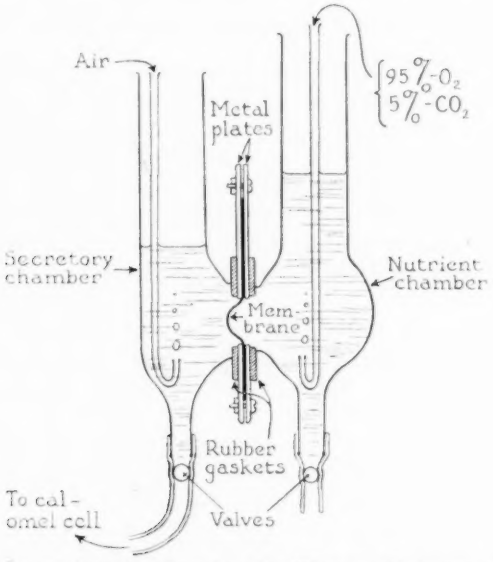

Fig. 1. Diagram of dual-chambered bath. When a pH determination is made, the aeration tube is removed and the glass electrode is inserted into the secretory chamber from the top.

the same solution as the secretory chamber (0.7 per cent NaCl). The junction between this solution and the reference cell was made by means of a KCl-agar cap as recommended by the Coleman Electric Company. The glass electrode was inserted into the secretory solution from the top, making it possible to record the pH at any time without withdrawing or contaminating the secretory solution. The pH readings were made with the Coleman pH Electrometer.

The nutrient solution had an ionic composition similar to that of frog's blood. Its high sodium bicarbonate content necessitated aeration with an oxygen-carbon dioxide mixture in order to maintain a pH of 7.4. The

solution contained, as millimols per liter, 71.75 of NaCl, 20.0 of $NaHCO_3$, 5.0 of KCl, 1.25 of $CaCl_2$, 1.20 of sodium phosphate buffer (pH 7.4), 0.80 of $MgCl_2$. Anhydrous glucose was added in a concentration of 50 mgm. per cent.

RESULTS. A large number of experiments have been made with various modifications of the apparatus and with various nutrient and secretory solutions. However, only results obtained with the method as described above, which yielded the most consistent and satisfactory results, will be given.

When the gastric mucosa of the frog was mounted as described, but not treated in any way, the pH of the secretory solution gradually fell over a period of hours and reached a plateau. In figure 2, the average of eight

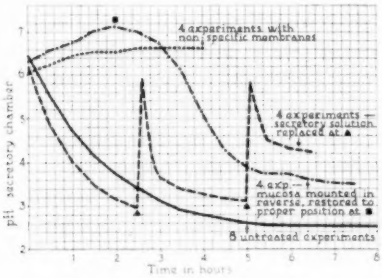
4 experiments with non-specific membranes
4 experiments — secretory solution replaced at ▲
4 exp. — 1 mucosa mounted in reverse, restored to proper position at ■
8 untreated experiments
pH secretory chamber
Time in hours

Fig. 2

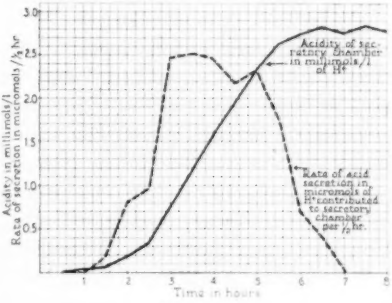
Acidity of secretory chamber in millimols/l of H+
Rate of acid secretion in micromols of H+ contributed to secretory chamber per ½ hr.
Acidity in millimols/l
Rate of secretion in micromols/½ hr
Time in hours

Fig. 3

Fig. 2. The *in vitro* secretion of acid. The changes in pH of the secretory chamber during 4 different types of experiment are given.

Fig. 3. The *in vitro* secretion of acid by the "untreated" mucosa. The acidity is given in millimols/l. (clinical units) and the output of acid each half hour is given in thousandths of a millimol.

consecutive experiments of this type is presented in the form of a graph. The pH fell from an original value of 6.4 to a final value of 2.55. The highest acidities of the individual experiments ranged from pH 2.86 to 2.14. The highest acidity that has been observed is pH 1.6.

In figure 3, the development of acidity in the secretory solution has been plotted in terms of millimols per liter, instead of pH units. The rate of acid secretion, in terms of micro-mols (thousandth of a milli-mol) of H^+ added to the bath each half-hour, is also shown. It is apparent that the mucosa does not begin to form appreciable amounts of acid until approximately two hours after being mounted, and that after 6 hours acid formation practically ceases.

The factors which are responsible for this "spontaneous" secretion have not yet been determined. The cessation of secretion, however, is not due

to an automatic suppression of further secretion by the presence of acid in the secretory solution. This is indicated by the fact that frequent renewal of the secretory solution does not alter the time at which secretion ceases (fig. 2).

Several types of experiment have yielded evidence which indicates that the *in vitro* production of acid represents a true secretory process. For example, non-specific membranes, such as the esophageal mucosa, the abdominal skin, or the abdominal musculo-peritoneal layer of the frog have failed without exception to form acid. In fact, the pH of the secretory solution has always increased (fig. 2). Of similar significance is our confirmation of Delrue (2) that gastric mucosa taken from winter frogs produces very little acid *in vitro*. It has been reported that the gastric glands of the intact frog do not secrete readily in winter (3).

Additional evidence that the *in vitro* production of acid is not the effect of a non-specific semi-permeable membrane was obtained in the following way. The gastric mucosa was mounted *in reverse* in the apparatus; the secretory surface was thus in contact with the nutrient solution and the muscularis surface in contact with the secretory solution. Under these conditions, in each of 14 experiments, the pH of the secretory solution rose. In fact, the solution became more alkaline than in the case of the non-specific membranes mentioned above, suggesting that alkali was being liberated from the muscularis surface. The fact that the pH of the buffered nutrient solution fell slightly supports this interpretation. After these membranes had been mounted in reverse for 2 hours, they were *restored* to the proper position. In ten cases the mucosa had apparently been killed and failed to secrete acid after this restoration; in four cases, however, the mucosa survived and produced significant amounts of acid (fig. 2).

Discussion. The results reported here confirm and extend the observations of Delrue. This investigator recorded the pH of the secretory solution by means of a quinhydrone electrode, which necessitated the periodic removal of samples for the determination of pH. By the use of the glass electrode, which does not contaminate the solution tested, this complicating factor has been avoided. The nutrient solution used in this study conforms more closely to the ionic composition of frog's blood than the solution used by Delrue. The technique employed has yielded consistent and uniform results.

In the majority of experiments Delrue (1) obtained pH values ranging from 4.2 to 4.8, although occasionally lower values (pH 2.$^+$) were obtained. This latter level he found to approximate the usual pH of the gastric contents of the frog. This was used as support for the belief that the *in vitro* acidity closely approximates the *in vivo* acidity of the frog's stomach. However, Friedman (4) has reported that pure gastric juice obtained

from the frog may have a free acidity of 72 millimols per liter, which represents a pH of 1.4. One could hardly expect to obtain this acidity *in vitro*, when the gastric secretion is diluted many times by the fluid of the secretory chamber.

In our experiments the acidity regularly fell considerably below the range of 4.2 to 4.8, for the average was 2.55. This difference is greater than appears superficially, due to the logarithmic nature of the pH unit. As can be seen from a comparison of the pH curve of figure 2 with the acidity curve of figure 3, a fall in pH from 6.0 to 4.0 represents an insignificant secretion of acid, whereas a fall from 3.5 to 2.5 represents the secretion of appreciable quantities of acid. In our experience, a pH below 3.0 has been followed by a gradual decrease in acidity after 8 to 10 hours. Delrue, on the other hand, reported that the plateau of acidity, presumably at higher pH levels, would persist for 24 hours or longer.

The production of sufficient acid to lower the pH of the secretory solution to an average value of 2.55, cannot be due to the formation of CO_2 by the dying mucosa. Irving (5) has shown that the pH of the gastric mucosa of the frog after death does not fall below pH 6.0. We have found that bubbling air through 0.7 per cent NaCl solution will not lower the pH below 6.0. If the formation of acid *in vitro* were a non-specific process, there is no reason why it should not have occurred when non-specific membranes were used, or when the mucosa was mounted in reverse.

CONCLUSIONS

1. The isolated gastric mucosa of the frog, when mounted *in vitro* in a suitable dual-chambered bath, formed sufficient acid to lower the pH of a saline solution to an average value of 2.55.

2. This formation of acid is believed to represent a genuine secretory activity on the part of the gastric glands for the following reasons: *a*, non-gastric membranes fail to form acid; *b*, gastric mucosa taken from winter frogs forms little acid; *c*, the gastric mucosa exhibits a polarity such that acid is secreted only from the natural secretory surface, and alkali is liberated only from the reverse side.

REFERENCES

(1) Delrue, G. Arch. inter. de Physiol. **33:** 196, 1930.
(2) Delrue, G. Arch. inter. de Physiol. **36:** 129, 1933.
(3) Friedman, M. H. F. J. Cell. and Comp. Physiol. **5:** 83, 1934.
(4) Friedman, M. H. F. J. Cell. and Comp. Physiol. **10:** 37, 1937.
(5) Irving, L. J. Cell. and Comp. Physiol. **2:** 1932.

INFLUENCE OF AGE ON KETOSIS

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The fasting rat developes a ketosis. The extent of this ketosis may be determined by many factors. Age appeared to be a factor of possible importance. Black, Collip and Thomson (1) noted a definite increase in the degree of ketosis with age when it was measured by the ketonuria of rats on a butter diet. Heymann (2) has examined the influence of age on ketosis in man by a study of the ketonuria at various ages on a ketogenic diet. He found that ketosis was minimal in infancy, increases during the first four years of life to a maximum which lasts until about eight years of age and then decreases until it reaches the adult level at puberty. We have found the excretion of acetone bodies in the urine to be an uncertain and misleading indicator of the degree of ketosis which exists. Consequently we have examined the relation of ketosis to age in the albino rat using the acetone body level of the blood as a measure of the degree of ketosis. This figure represents the difference in the rate of production of acetone bodies by the liver and their rate of oxidation by the extrahepatic tissues.

EXPERIMENTAL. Male albino rats of known age were used. They had been receiving as a stock diet "Tioga dog pellets" (protein, 23.0; fat, 4.0; fiber, 4.0; ash, 12.5; moisture, 8.5; nitrogen-free extract, 48.0) and were fasted directly from this diet. Fasting was commenced at 9 o'clock in the evening but since rats generally sleep all day and eat at night they had already in one sense been some 12 hours without food when fasting was commenced. At twelve hour intervals groups were sacrificed for measurement of the acetone body level in the blood. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital and a blood specimen taken by severing the abdominal aorta. Total acetone bodies in the blood samples were determined by the method of Barnes and Wick (3) and liver glycogen by the method of Good, Kramer and Somogyi (4). Each group contained 3 rats except the 30 day old series in which there were six per group.

RESULTS. Averages of all pertinent data are presented in table 1. Following the onset of fasting at any age there is an increase in the level of acetone bodies in the blood. This increase tends to take place more

TABLE 1

The influence of age on the degree of fasting ketosis and the amount of glycogen in the liver

Group averages

AGE	HOURS OF FASTING								
	0	12	24	36	48	60	72	84	94
Blood acetone body concentration in milligrams per cent									
days									
30	0.3	15	25	29	23				
45		10	17	19	18	23	16	20	19
61	1.0	14	19	22	22	26	19	28	21
76	1.1	8	16	18	22	22	26	19	25
128	0.8	6	12	11	23	17	20	20	16
275	0.8	5	9	10	14	24	23	17	20
Liver glycogen concentration in grams per cent									
30	1.73	0.23	0.03	0.18	0.06				
45	2.90	0.17	0.04	0.13	0.36	0.51	0.57	0.43	0.30
61	2.00	0.11	0.04	0.11	0.25	0.21	0.06	0.33	0.11
76	2.80	0.34	0.03	0.13	0.05	0.31	0.10	0.75	0.15
128	3.30	0.79	0.09	0.13	0.05	0.36	0.16	0.53	0.43
275	1.80	0.66	0.06	0.19	0.18	0.13	0.05	0.23	0.19
Liver glycogen in milligrams per square decimeter body surface									
30	163.0	20.0	2.8	11.7	4.7				
45	160.0	9.2	2.0	6.7	15.8	24.0	23.7	18.1	12.1
61	109.0	5.8	1.8	5.0	12.2	9.7	2.3	14.0	4.3
76	176.0	18.9	1.5	6.8	2.4	15.7	4.3	39.0	6.2
128	196.0	26.0	3.3	5.7	2.4	14.9	6.3	22.0	11.3
275	110.0	34.0	2.7	8.7	8.7	6.4	1.9	8.1	8.1
Urine nitrogen in milligrams per square decimeter body surface per day									
30		27		31*					
45		31		26*		30*			
61		31		32		23*		24	
76		31		29		25*		21*	
128		26		23*		20*		23	
275		35		25*		19*		18*	
Liver weight in grams									
30	3.11	2.75	2.43	1.95	2.19				
45	5.16	4.83	4.00	4.00	3.53	3.66	3.33	3.10	3.16
61	6.46	5.44	4.90	5.00	5.13	4.66	4.20	4.30	4.00
76	7.97	7.16	6.17	6.35	5.66	5.89	5.00	5.94	4.76
128	8.65	7.05	5.55	5.59	5.71	5.68	5.38	5.52	5.15
275	11.21	9.66	8.74	8.43	8.74	8.55	7.44	7.51	7.60
Body weight in grams									
30	72	67	65	61	58				
45	121	112	106	99	97	92	96	87	90
61	169	158	156	148	147	142	136	137	132
76	199	195	189	183	182	174	173	167	166
128	241	232	226	221	216	215	207	206	198
275	348	341	336	333	327	322	321	316	316

rapidly, the younger the rat, but after the first 48 hours of fasting there is no significant difference in the degree of fasting ketosis at any age when it is measured by the blood acetone body level.

Urine collections were made over 24 hour periods primarily for nitrogen determinations. None of the specimens contained acetone bodies in measurable quantities but traces were present in all samples which in the nitrogen figures in table 1 are marked with an asterisk.

The changes in the amount of glycogen in the liver do not follow the variations in the acetone body concentration in the blood except for the tendency of the glycogen to fall more rapidly in young than in old animals. In all cases it reached a minimum after 24 hours of fasting and then tended to rise, in some cases increasing to significant amounts.

Discussion. The decrease in the rate of onset of ketosis as the rats are older is undoubtedly due to the greater glycogen stores available for energy in the older rats. As these are exhausted and the organism is dependent on protein alone as a source of sugar the formation of acetone bodies reaches a common level. This suggests that after the first two days of fasting the relationship of the protein to fat which is utilized on further fasting must be the same at all ages. The urine nitrogen figures which are all of the same order of magnitude support this conclusion.

SUMMARY

The fasting ketosis of male albino rats as measured by the level of acetone bodies in the blood increases less rapidly the older the rat. After 48 hours of fasting it has reached a similar maximum for all ages.

The amount of glycogen in the liver tends to decrease somewhat less rapidly on fasting in old than in young rats. At all ages it reaches a minimum after 24 hours without food and then tends to increase for the next three days.

REFERENCES

(1) Black, P. T., J. B. Collip and D. L. Thomson. J. Physiol. **82:** 385, 1934.
(2) Heymann, W. J. Pediatrics **12:** 21, 1938.
(3) Barnes, R. H. and A. N. Wick. J. Biol. Chem. **131:** 413, 1939.
(4) Good, C. A., H. Kramer and M. Somogyi. J. Biol. Chem. **100:** 485, 1933.

THE EFFECTS OF ANGIOTONIN ON RENAL BLOOD FLOW AND GLOMERULAR FILTRATION

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Purified renin, the so-called renal pressor substance, does not cause vasoconstriction when perfused through the isolated rabbit's ear (Page, 1939). Addition of "renin-activator" (Kohlstaedt, Page and Helmer, 1940), a substance obtained from blood plasma, to solutions of renin permits full vasoconstrictor effect. Further study has shown that a crystalline pressor substance is obtained by the interaction of renin and renin-activator (Page and Helmer, 1940). This substance has been called angiotonin and is probably the effector substance of the action of renin.

Renin, when slowly infused intravenously into conscious, trained dogs, causes a prolonged decrease of renal blood flow which is in great part the result of efferent glomerular vasoconstriction (Corcoran and Page, 1939a, 1940). The renal action of angiotonin was therefore examined by similar methods. It was expected that the action of angiotonin would in some respects differ from that of renin, since its liberation into the blood would probably be slower and more uniform when presented by renin infusion than when given as such. A general similarity of renal effects would support the view that renin acts by liberation of angiotonin.

METHODS. The methods used were those previously described in detail (Corcoran and Page, 1939). Renal blood flow (C/Ep 100), phenol red and inulin clearances $\left(C = \frac{UV}{P}\right)$, and renal extraction percentages $\left(\frac{A\text{-}V}{A}\ 100\right)$ were determined in trained, uninephrectomized female dogs whose remaining kidney had been subcutaneously explanted by the method of Page and Corcoran (1940). Plasma and urine phenol red and inulin concentrations were found by the methods of Corcoran and Page (1939a, b).

Each experiment consisted of two or more control periods, during which phenol red and inulin in saline solution were infused at the rate of 1 cc. per minute, their renal clearances and extractions determined, and renal blood and plasma flows found by calculation. Angiotonin, prepared by Dr. O. M. Helmer, was then added to the infusing fluid, and the observa-

tions continued. Blood pressures were recorded once in each period by femoral arterial puncture.

Results. The results of two experiments are presented graphically (fig. 1). The protocols of these and of 3 other experiments are tabulated at the end of this paper. Several experiments done with weak solutions of angiotonin shortly after its existence was recognized resulted in only transient increases of arterial pressure and fleeting renal responses. These are not included in this report. Experiments done with larger doses of angiotonin, in which sustained increases of arterial pressure were observed,

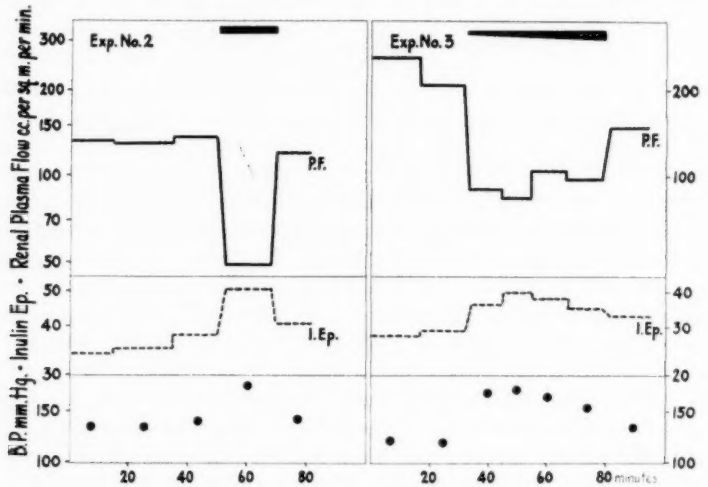

Fig. 1. Effect of infusion of angiotonin on renal plasma flow, inulin extraction percentage and femoral arterial blood pressure. *P. F.*, renal plasma flow; *I. Ep.*, inulin extraction percentage; *B. P.*, femoral arterial pressure millimeters of mercury. Period of angiotonin infusion indicated by shaded area.

always resulted in decreased renal blood flow and increased renal extraction of inulin.

The infusion of angiotonin at a steady pace reduced renal blood flow, increased arterial pressure and renal extraction of insulin. Interruption of the angiotonin infusion promptly results in a return of blood pressure, renal blood flow, and renal extraction towards normal.

It will be noted that renal extraction of phenol red is relatively high during angiotonin infusion, even at plasma levels at which the load of phenol red brought in the plasma to the renal secretory cells is so large that the proportion removed from blood ordinarily decreases (above 1.5 mgm. per 100 cc.). We have previously observed increased renal extraction of phenol red during renal ischemia at plasma levels below 1.5 mgm.

TABLE 1

Effects of angiotonin infusion on renal plasma flow, phenol red and inulin clearances and extraction percentages

Time in minutes from each emptying of the bladder. Angiotonin—relative amounts infused indicated by plus sign. P.R.C., plasma phenol red clearance; P.R.Ep., phenol red plasma extraction percentage; P.P.R., arterial plasma phenol red in milligrams per 100 cc.; I.C., plasma inulin clearance; I.Ep., plasma inulin extraction percentage; P.F., renal plasma flow; mean of values calculated from phenol red and inulin clearances and extractions; B.P., mean femoral arterial pressure in millimeters of mercury. Values for renal clearance and plasma flow expressed as cubic centimeters per square meter body surface per minute. Decimal fractions used in the calculation of renal plasma flow are not included in the table.

EXPERIMENT NUMBER	TIME	ANGIOTONIN	P.R.C.	P.R.EP.	P.P.R.	I.C.	I.EP.	P.F.	B.P.
1	0–18	0	59	50	0.872	35	26	126	115
	18–39	0	50	49	0.828	32	33	98	108
	42–64	+	20	78	2.46	19	44	34	158
	64–79	+	53	54	3.12	34	44	86	148
2	0–15	0	92	64	0.530	42	34	133	134
	15–35	0	82	59	0.623	43	35	130	133
	35–50	0	97	65	0.865	48	38	136	140
	53–68	++	32	77	2.79	29	51	49	187
	70–82	0	71	59	4.61	45	41	115	141
3	0–17	0	131	49	0.670	72	28	262	121
	17–32	0	94	50	0.820	67	29	207	118
	34–45	+	50	63	1.27	45	36	91	167
	45–55	++	40	56	1.95	38	40	85	171
	55–67	++	62	52	2.14	36	38	106	160
	67–79	+++	55	63	2.81	49	35	99	155
	82–95	0	74	51	2.23	52	33	150	134
4	0–19	0	87	45	0.860	62	34	186	121
	19–35	0	89	43	0.913	64	33	200	118
	35–52	0	75	43	0.958	53	32	172	120
	55–67	+	36	60	2.01	39	42	76	171
	67–79	+	47	54	2.92	59	44	110	160
	79–91	+	51	57	3.84	53	44	104	155
5	0–13	0	113	52	0.692	55	28	204	124
	13–27	0	135	59	0.650	67	28	235	121
	27–45	0	99	47	0.461	56	27	212	132
	48–59	+	61	54	0.753	34	46	100	142
	59–75	+	70	55	0.822	26	36	99	139

per 100 cc. and have attributed this effect to a relative delay of the blood stream in the peritubular capillaries. The increased renal extraction of

phenol red observed during the renal ischemia produced by angiotonin has probably a similar mechanism.

As with renin (Landis, Montgomery and Sparkman, 1938), infusion of angiotonin causes no consistent change in skin temperature, which was measured in a few experiments. Large doses caused salivation, and occasionally retching, which were also observed with large doses of renin.

Discussion. Infusion of angiotonin causes renal ischemia which, at least at the start of the infusion, may be profound, although it tends to lessen as the infusion is continued. The degree of ischemia is relatively greater than that observed during infusion of renin in doses which produced the same percentile increase of arterial pressure. The action of angiotonin seems also more fleeting than that of renin, since both renal blood flow and arterial pressure tend promptly to return towards normal after interruption of the infusion, although, as with renin (Merrill, Williams and Harrison, 1938), the return of renal blood flow lags behind the return of arterial pressure. These observations are consistent with the action of a substance which, when presented during renin infusion, is slowly liberated within the body, and, when presented as such, acts more intensely and ceases acting as soon as it is withdrawn because there is no circulating source of the material as there is when renin has been given.

The increased renal extraction of inulin observed during angiotonin infusion is similar to that observed during infusion of renin. Removal of inulin from blood in the kidney occurs by glomerular filtration from the plasma water, and variations in its removal must result from variations of intraglomerular pressure. Since arterial hypertension, presumably due to increased cardiac output, has relatively little effect on intraglomerular pressure as measured by inulin extraction (Corcoran and Page, 1939a), the changes observed during angiotonin infusion occurred as the result of circulatory adjustments within the kidney, either by dilatation of the afferent or constriction of the efferent arterioles. The co-existence of renal ischemia with increased intraglomerular pressure strongly suggests that the increased filtration pressure, and, in some measure at least, the restriction of renal blood flow occurred as the result of constriction of glomerular efferent arterioles. Since in some experiments as much as 45 per cent of the arterial plasma inulin (and water) was removed in the kidney by filtration, the increase in intraglomerular pressure must have been very great.

The renal action of angiotonin is, then, similar to that of renin and differs only in those characteristics by which one would expect an effector substance to differ from a material from which it is slowly liberated. The renal effects of angiotonin and renin are similar to the mechanisms which obtain in arterial hypertension in human beings (Smith, Goldring, Chasis and Ranges, 1938; Goldring, Chasis, Ranges and Smith, 1938), namely, decreased renal blood flow and increased filtration fraction (renal extrac-

tion of inulin), both, apparently, due to efferent arteriolar constriction. It is of interest to note from the observations of Smith and colleagues and from our own unpublished data that renal extraction of phenol red is also increased in these cases, probably by a mechanism similar to that operating during the infusion of angiotonin and renin.

SUMMARY

Infusion of angiotonin into conscious, trained dogs with explanted kidneys, results in increased arterial pressure, decreased renal blood flow, and increased extraction of inulin from blood by the kidney. These effects are similar to those observed during infusion of renin, although with angiotonin the effects were initially more profound, and, after interruption of the infusion, more fleeting. The increased extraction of inulin and restriction of renal blood flow which occur during angiotonin infusion are, in great part, the result of efferent glomerular vasoconstriction. The effects of angiotonin on the kidney support the view that it is the effector substance of renin action.

REFERENCES

Corcoran, A. C. and I. H. Page. This Journal **126:** 354, 1939a.
J. Biol. Chem. **127:** 601, 1939b.
This Journal **129:** 698, 1940.

Goldring, W., H. Chasis, H. A. Ranges and H. W. Smith. J. Clin. Investigation **17:** 505, 1938.

Kohlstaedt, K. G., I. H. Page and O. M. Helmer. Am. Heart J. **19:** 92, 1940.

Landis, E. M., H. Montgomery and D. Sparkman. J. Clin. Investigation **17:** 189, 1938.

Merrill, A., R. H. Williams and T. R. Harrison. Am. J. Med. Sci. **196:** 240, 1938.

Page, I. H. J. Exper. Med. **70:** 521, 1939.

Page, I. H. and A. C. Corcoran. Surgery **7:** 389, 1940.

Page, I. H. and O. M. Helmer. J. Exper. Med. **71:** 29, 1940.

Smith, H. W., W. Goldring, H. Chasis and H. A. Ranges. This Journal **123:** 189, 1938.

OSMOTIC RELATION BETWEEN AQUEOUS HUMOR AND BLOOD PLASMA[1]

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Gilman and Yudkin (1933), using the Hill (1930) method of measuring vapor pressure, found the aqueous humor of dogs to be isosmotic to the blood. Benham, Davson, and Duke-Elder (1937), using Baldes' (1934) modification of the thermoelectric method, found the aqueous humor of cats under ether anesthesia to be hyposmotic to the blood serum. However, in a more recent study, Benham, Duke-Elder and Hodgson (1938) found that under local anesthesia the aqueous humor was hyperosmotic to blood serum and concluded that aqueous humor was formed by secretion rather than by filtration. They explained their earlier results as being due to the fact that ether anesthesia results in an increase in the osmotic activity of the blood.

Investigators who have studied the distribution of ions between blood and aqueous humor also disagree as to the mechanism of aqueous humor formation. Hodgson (1938) finds the ratio of chloride in the aqueous humor to that in the blood serum to be greater than that expected on the basis of ultrafiltration. Davson (1939) believes that the distribution of sodium and chloride between serum and aqueous humor does not justify the assumption of a secretory mechanism for the formation of aqueous humor. Walker (1933) found the concentration of urea in the aqueous humor of rabbits, dogs and man to be on the average 30 per cent less than in the plasma. Similar differences were observed in the concentration of uric acid in the aqueous humor and plasma of fowls and man and in the concentrations of inorganic phosphates and reducing substances in the aqueous humor and plasma of frogs. From these results Walker concluded that aqueous humor is not formed by simple filtration or dialysis.

In attempting to throw further light on the mode of formation of aqueous humor, we have repeated some of the work of Benham et al. (1938) and, in addition, we have studied the osmotic relationship between aqueous humor and blood plasma following the injection of a poison, $HgCl_2$, into the anterior chamber of the eye.

[1] This work has been aided by grants from the Rockefeller Foundation and the Medical Research Fund of the University of Minnesota to Prof. M. B. Visscher.

EXPERIMENTAL PROCEDURE. Determinations of osmotic activity were made by the thermoelectric method as modified by Baldes (1934) and Baldes and Johnson (1939). All determinations were made in the presence of 5 per cent CO_2 in oxygen and the results are expressed in terms of the concentration of an isosmotic solution of NaCl. Blood samples were drawn in an oiled syringe from the femoral artery. The blood was trans-

TABLE 1

Comparison of osmotic activity of aqueous humor and blood plasma with animals under Nembutal anesthesia

ANIMAL	TIME	OSMOTIC ACTIVITY IN mM NaCl PER KILO H_2O		
		Plasma	Aqueous humor	Difference
	minutes			
Rabbit 1		155.6	158.5	+2.9
Rabbit 2		164.7	165.9	+1.2
Rabbit 3	0	147.2	148.6	+1.4
	32	147.6	150.0	+2.4
Rabbit 4	0	164.0	165.8	+1.8
	32	163.7	165.3	+1.6
Average, 4 rabbits				+1.9
Dog 1	0	160.6	R 164.0	+3.4
	27	160.9	L 164.9	+4.0
	72	161.0	R 165.3	+4.3
Dog 2	0	160.6	R 163.3	+2.7
	54	159.8	L 163.7	+3.9
Dog 3	0	160.0	R 164.0	+4.0
	37	159.3	R 160.9	+1.6
	79	159.0	R 162.3	+3.3
Dog 11	0	161.3	L 164.7	+3.4
	29	163.2	L 166.1	+2.9
	93	164.4	L 166.7	+2.3
Average, 4 dogs				+3.3

ferred to a cooled test tube containing oil and a small amount of dried heparin (Connaught), the tube was stoppered and the plasma separated immediately by centrifugation. Samples of aqueous humor were removed with a tuberculin syringe by lateral insertion into the anterior chamber.

In adding $HgCl_2$ to the aqueous humor in the anterior chamber, a measured volume (0.01–0.05 cc. as indicated in the table of results) of a 0.1 M solution of $HgCl_2$ was placed on the end of the plunger of a 2 cc.

syringe and evaporated to dryness. A portion of the aqueous humor (0.3–0.8 cc.) was then drawn into the syringe, the $HgCl_2$ dissolved by rotating the syringe and the solution reinjected into the anterior chamber. Nembutal was used as the general anesthetic and procain (3 per cent solution) as the local anesthetic.

RESULTS AND COMMENT. The results obtained by comparing the osmotic activity of aqueous humor and blood plasma of rabbits and dogs under Nembutal are given in table 1. In every instance the osmotic activity of the aqueous humor was greater than that of the blood plasma. Since changes in the osmotic activity of aqueous humor would necessarily lag behind any changes occurring in the blood plasma, several series of sam-

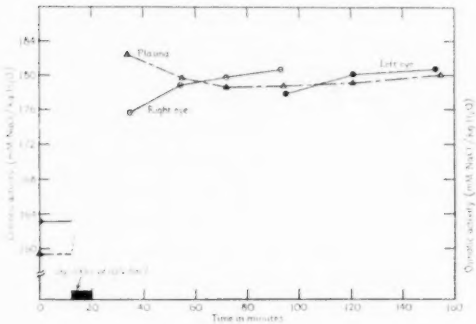

Fig. 1

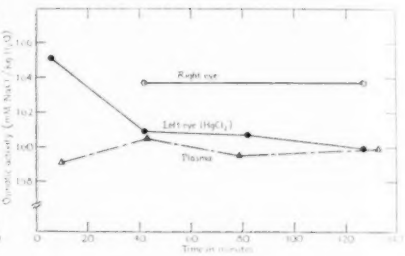

Fig. 2

Fig. 1. Changes in osmotic activity of aqueous humor and blood plasma of dog following intravenous injection of 5.0 cc. of 10 per cent NaCl per kilo of body weight. (Kidneys ligated.)

Fig. 2. Osmotic relation between aqueous humor and blood plasma of dog. Seven-tenths cubic centimeter of aqueous humor removed from left eye and reinjected with 0.003 milli-mol $HgCl_2$ posterior to iris.

ples were collected over a period of time in order to be sure that the osmotic activity of the plasma was relatively constant. Since only 0.05–0.10 cc. of aqueous humor was removed for each determination, several samples could be taken from the same eye.

Changes in the osmotic activity of aqueous humor and blood plasma following the intravenous injection of hypertonic NaCl are shown in figure 1. In this experiment the kidneys were ligated in order that the osmotic activity of the plasma would remain constant at the elevated level. It should be noted that if the volume of aqueous humor in the eye is not reduced by the removal of samples, more than 80 minutes is required for the aqueous humor to become again hyperosmotic to the blood plasma.

It seemed possible that excitement of the animal accompanying the

withdrawal of aqueous humor from untrained dogs under local anesthesia might result in an increase in the osmotic activity of the plasma above that of the aqueous humor. With this in mind an attempt was made to duplicate the results of Gilman and Yudkin (1933) who obtained their samples under local anesthesia. The results of these experiments are given in table 2. It was found that under local anesthesia the difference in osmotic activity between aqueous humor and plasma is even greater than under Nembutal anesthesia. With dogs 7 and 8 two samples of blood were taken, one before and the other immediately after the removal of the aqueous humor. With dogs 9 and 10, the blood samples were taken immediately following the removal of the aqueous humor and in these experiments the dogs became excited and struggled appreciably while the

TABLE 2

Comparison of osmotic activity of aqueous humor and blood plasma of dogs under local anesthesia (Procaine)

DOG NO.	OSMOTIC ACTIVITY IN mM NaCl PER KILO H_2O		
	Blood plasma	Aqueous humor	Difference
7	158.0 157.3	162.8	+5.1
8	153.3 152.9	159.8	+6.7
9	154.7	161.0	+6.3
10	152.8	156.3	+3.5
Average			+5.4

samples were being taken. Dog 10 was recovering from distemper at the time of the experiment.

Although the osmotic activity of the aqueous humor is found to be consistently higher than that of the blood plasma, the question arises as to whether this difference, as measured, actually exists in vivo. One may not be justified in assuming that the blood removed from the femoral artery has the same osmotic activity as the blood flowing through the optic artery. In order to show more conclusively that the difference in osmotic activity between aqueous humor and blood plasma is real and is indicative of an active secretory process, we studied the effect of injecting $HgCl_2$ into the anterior chamber upon this difference in osmotic activity. As shown by the data given in table 3 and the graph in figure 2, the effect of $HgCl_2$ is to reduce or abolish the difference in osmotic activity between the plasma and aqueous humor. In most of the experiments the aqueous humor and

$HgCl_2$ were injected into the anterior chamber posterior to the iris since the injection of $HgCl_2$ anterior to the iris does not appear to be as effective in reducing the difference in osmotic activity. This is shown by the results obtained with dogs 3 and 6, and is probably due to the circulation of aqueous humor from the ciliary bodies posterior to the iris into the chamber anterior to the iris (Best and Taylor, 1939).

The concentration of $HgCl_2$ in the aqueous humor necessary to abolish the difference in osmotic activity is of the same order of magnitude although slightly higher than that found by Ingraham and Visscher (1936) to poison the absorption of chloride from the ileum of dogs.

TABLE 3

The effect of injection of $HgCl_2$ into the anterior chamber of the eye on the difference in osmotic activity between aqueous humor and blood plasma

DOG	TIME	OSMOTIC ACTIVITY IN mM NaCl PER KGM. WATER				
		Plasma	Aqueous humor		Difference	
			Left eye	Right eye	Left eye	Right eye
	*minutes**					
3	35	160.0	161.9†	164.0	+1.9	+4.0
	73	159.3	160.6	160.9	+1.3	+1.6
	114	159.0	160.9	162.3	+1.9	+3.3
4	135	158.9	158.9‡	163.7	0.0	+4.8
5	90	159.1	160.5§	163.9	+1.4	+4.8
6	160	160.4	160.8¶	161.4‖	+0.4	+1.0
12	100	157.1	157.2**	160.6	+0.1	+3.5
	142	158.1	156.6	160.8	−1.5	+2.7

* Time following injection of $HgCl_2$ into anterior chamber of left eye.

† 0.8 cc. of aqueous humor removed and reinjected with 0.0025 milli-mols $HgCl_2$ anterior to iris.

‡ 0.7 cc. of ditto with 0.003 milli-mol $HgCl_2$ posterior to iris.

§ 0.5 cc. of ditto with 0.001 milli-mol $HgCl_2$ posterior to iris.

¶ 0.3 cc. of ditto with 0.001 milli-mol $HgCl_2$ posterior to iris.

‖ 0.5 cc. of ditto with 0.005 milli-mol $HgCl_2$ anterior to iris.

** 0.5 cc. of ditto with 0.002 milli-mol $HgCl_2$ posterior to iris.

These results indicate that aqueous humor is not an ultrafiltrate of blood plasma. In the formation and circulation of aqueous humor there appears to be a net transport of a hyperosmotic solution from the blood plasma into the anterior chamber of the eye. This mechanism may be similar to that involved in the absorption of chloride from the intestine where differences in total osmotic activity also occur. It has been shown that during the absorption of chloride from the ileum against a concentration gradient, the osmotic activity of the intestinal fluid decreases below that of the blood plasma (Roepke and Visscher, 1939). In that case there is a net movement of hyperosmotic solution into the blood.

The authors are indebted to Prof. M. B. Visscher for his helpful suggestions and criticisms.

SUMMARY

The osmotic activity of aqueous humor was found normally to be higher than that of the blood plasma. The re-injection of aqueous humor containing $HgCl_2$ into the anterior chamber posterior to the iris was found to abolish the difference in osmotic activity between aqueous humor and blood plasma.

The results support the view that active secretory processes are involved in the formation of aqueous humor.

REFERENCES

BALDES, E. J. J. Sci. Instruments **11:** 223, 1934.
BALDES, E. J. AND A. F. JOHNSON. Biodynamics **47:** 1, 1939.
BENHAM, G. H., H. DAVSON AND W. S. DUKE-ELDER. J. Physiol. **89:** 61, 1937.
BENHAM, G. H., W. S. DUKE-ELDER AND T. H. HODGSON. J. Physiol. **92:** 355, 1938.
BEST, C. H. AND N. B. TAYLOR. The physiological basis of medical practice. Baltimore, Williams & Wilkins Co., 1939.
DAVSON, H. J. Physiol. **96:** 194, 1939.
GILMAN, A. AND A. M. YUDKIN. This Journal **104:** 235, 1933.
HILL, A. V. Proc. Roy. Soc. A. **127:** 9, 1930.
HODGSON, T. H. J. Physiol. **94:** 118, 1938.
INGRAHAM, R. C. AND M. B. VISSCHER. This Journal **114:** 681, 1936.
ROEPKE, R. R. AND M. B. VISSCHER. Proc. Soc. Exper. Biol. and Med. **41:** 500, 1939.
WALKER, A. M. J. Biol. Chem. **101:** 269, 1933.

ANALYSIS OF THE VASOPRESSOR AND OTHER "NICOTINIC" ACTIONS OF ACETYLCHOLINE

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Hunt (1) observed that large doses of acetylcholine caused a rise rather than a fall in blood pressure in animals in which the vasodilator action of this drug was opposed by atropine. Numerous other early authors (2, 3, 4, 5, 6 and 7) described the vasopressor effect of choline and some of its derivatives in atropinized animals. Dale (7) distinguished between the "muscarine-like" (vasodilator and other parasympathomimetic actions) and the "nicotine-like" effects of acetylcholine. He assumed that the latter effects were due to a ganglionic stimulant action of acetylcholine and showed that doses of nicotine sufficient to paralyze autonomic ganglia abolished the vasopressor effects of acetylcholine.

More recently, Simonart (8), and Feldberg and Minz (9) confirmed the observations of Dale and others, but believed that at least part of the acetylcholine rise was due to mobilization of epinephrine from the suprarenal glands.

The assumption of Hunt and Dale that the vasopressor action of acetylcholine is due to ganglionic stimulation received confirmation by the work of Feldberg and Gaddum (10), and by Koppanyi, Dille and Linegar (11). The latter have shown that the depression or paralysis of the superior cervical ganglion by nicotine may be overcome by large doses of acetylcholine or physostigmine. The present work was undertaken to extend these observations to the vasomotor sympathetic ganglia and to analyze the vasopressor and other "nicotine-like" actions of acetylcholine.

EXPERIMENTAL. Dogs and cats were used throughout the experiments. In most cases sodium pentobarbital (30–35 mgm.[1]) administered intravenously was used as an anesthetic; in others sodium amytal (50 mgm. by vein) or urethane (from 0.75–1.00 gram intraperitoneally) was used. The drugs used were administered intravenously in all cases with the exception of cocaine hydrochloride, which was also given intramuscularly.

[1] All doses are expressed in terms of milligrams per kilogram of body weight, unless otherwise stated. To avoid repetition the words "per kilogram of body weight" are omitted.

In all animals the blood pressure was recorded from the common carotid artery and the respiration by connecting a tracheal cannula with a Marey tambour. Duodenal motility was recorded with a water manometer connected to a balloon inserted into the upper duodenum through the stomach.

In several experiments it was necessary to inject fixed quantities of arterial blood of one animal into a vein of another. This was done by inserting a three-way glass cannula into the common carotid artery of the donor and into the femoral vein of the recipient (dogs or cats) with each cannula connected by small rubber tubing to a metal three-way T-valve to which a 30 cc. syringe was attached. A clamp was placed on the cannulated carotid artery between injections. By releasing the clamp on the artery and opening the valve of the metal T-tube on the arterial side, the syringe was allowed to fill to the desired volume, the arterial valve was closed, the valve connected with the femoral vein opened, and the blood injected into the recipient at a constant rate. Careful cleaning of the cannulae and prompt saline infusion following the transfusion made it unnecessary to use an anticoagulant.

A. *The action of physostigmine on the acetylcholine pressor effect.* It is a well known fact that doses of acetylcholine from 1 to 5 mgm. produce a marked vasopressor effect in atropinized animals. In these animals (2 to 5 mgm. of atropine sulfate) under barbiturate or urethane anesthesia we have found that 0.5 mgm. of acetylcholine chloride usually produced pressor effects, but that 0.15 mgm. or less did not produce pressor responses except for small rises in an occasional animal. It was observed, however, that when an atropinized animal was given 0.5 to 1.0 mgm. of physostigmine salicylate or prostigmin, acetylcholine in doses of 0.025 to 0.1 mgm. produced sharp epinephrine-like rises in blood pressure of 30 to 120 mm. of mercury (see fig. 1). Occasionally, with optimum amounts of physostigmine, definite pressor effects were seen following the injection of 0.001 to 0.005 mgm. of acetylcholine.

In animals in which the amounts of atropine (5.0 mgm.) and acetylcholine (0.05 mgm.) were being kept constant, physostigmine was administered in gradually increasing amounts from 0.005 mgm. upward. The results showed that in the average animal from 0.03 to 0.04 mgm. of physostigmine was necessary to obtain a definite elevation of blood pressure from 0.05 mgm. of acetylcholine. Doses of 0.02 mgm. of physostigmine or less were ineffective, while with up to 0.5 mgm. of physostigmine the magnitude of the acetylcholine pressor effect increased. With 0.5 to 1.5 mgm. of physostigmine the acetylcholine vasopressor effects were about optimal, while beyond that point they began to decline. Artificial respiration was often necessary after the animal had received 4 to 6 mgm. of physostigmine or 1 to 2 mgm. of prostigmin.

The doses of atropine used in all experiments varied from 0.5 to 60 mgm. In general large doses of atropine did not appear to interfere either with the vasopressor action of acetylcholine or the action of physostigmine, unless it produced toxic effects with marked fall in blood pressure.

In addition to acetylcholine two other choline derivatives with "nicotine-like" action, viz., muscarine (nitrous acid ester of choline) and doryl (carbamyl choline chloride), were given in doses of 0.05 to 1 mgm. to atropinized animals. The minimum dose of muscarine producing a rise in blood pressure in atropinized dogs lies between 0.1 to 0.5 mgm. and of doryl 0.05 to 0.2 mgm. One-half to 1.0 mgm. of muscarine produced

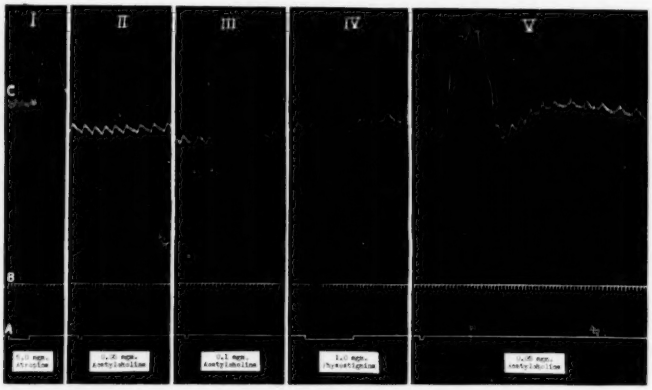

Fig. 1. Dog, 9.6 kgm., 30 mgm. of sodium pentobarbital per kilogram by vein. A, line indicating injection of drugs. B, time, 2 seconds; base line representing zero mm. of Hg pressure. C, blood pressure tracing from the common carotid artery. I, 5.0 mgm. of atropine sulfate per kilogram, vein. II, two minutes later; 0.05 mgm. of acetylcholine chloride per kilogram, vein. III, one minute later; 0.1 mgm. of acetylcholine chloride per kilogram, vein. IV, one minute later; 1.0 mgm. of physostigmine salicylate per kilogram, vein. V, five minutes later. 0.05 mgm. of acetylcholine chloride per kilogram, vein.

pressor effects ranging from 30 to 70 mm. of mercury, and 0.5 to 1 mgm. of doryl rises in blood pressure of 40 to 120 mm. Physostigmine in doses of 0.1 to 5 mgm. did not increase the magnitude of the pressor responses of either muscarine or doryl.

B. *Phenomena accompanying the pressor effect of acetylcholine.* In addition to the elevation of blood pressure, the animals treated with atropine and physostigmine show central stimulation, increase in respiration (16), stimulation of the ocular sympathetics, relaxation of the gut and cardiac acceleration following the injection of 0.05 mgm. of acetylcholine.

In a number of experiments in which the duodenal motility was recorded,

acetylcholine in the presence of small amounts of atropine and relatively large amounts of physostigmine produced a distinct phase of relaxation of the intestine (see fig. 2). In those cases in which the pressor effect of acetylcholine was preceded by a marked fall in blood pressure, this intestinal relaxation occurred before the blood pressure elevation. In control experiments when pressor responses were produced by rapid injection of 7 per cent acacia solution there was no effect on intestinal motility and when blood pressure falls were produced by injections of acetyl-beta-methylcholine there was only an increase in duodenal activity (see fig. 2).

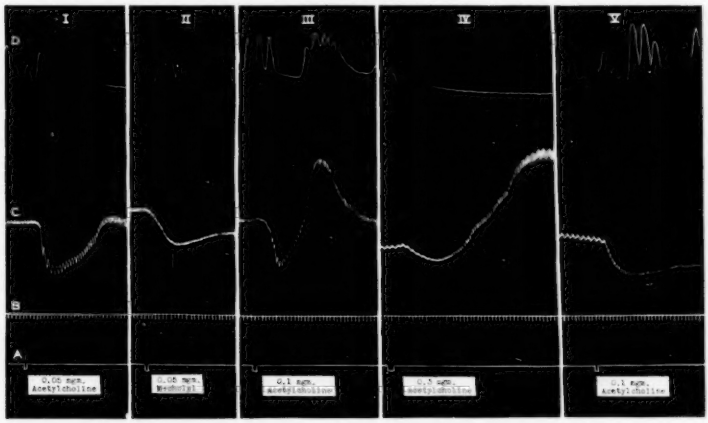

Fig. 2. Dog, 8.6 kgm., 35 mgm. of sodium pentobarbital, 0.1 mgm. of atropine sulfate and 0.5 mgm. of prostigmin per kilogram by vein.

A, line indicating injection of drugs. B, time, 2 seconds; base line representing zero mm. of Hg pressure. C, blood pressure tracing from the common carotid artery. D, tracing of duodenal motility.

I, 0.05 mgm. of acetylcholine chloride. II, 0.05 mgm. of acetyl-beta-methylcholine chloride. III, 0.1 mgm. of acetylcholine chloride. IV, 0.3 mgm. of acetylcholine chloride. V, following 3.0 mgm. of nicotine salicylate; 0.1 mgm. of acetylcholine chloride.

Nicotine in doses of 1 to 10 mgm. abolished the pressor effect of acetylcholine and the accompanying intestinal relaxation.

C. *The duration of action of physostigmine in atropinized animals.* In two dogs receiving sodium pentobarbital, the duration of action of 1.0 mgm. of physostigmine upon the vasopressor effect of 0.05 mgm. of acetylcholine following 3.0 mgm. of atropine was found to be about six hours. The acetylcholine effects did not diminish in height until about the end of the physostigmine action, but the latent period between injection of acetylcholine and the beginning of the blood pressure rise was lengthened,

and the rise in blood pressure was more gradual though of the same height. At the end of physostigmine action acetylcholine produced a small rise, no rise, or even a fall in blood pressure. Additional physostigmine restored the pressor effects without the necessity of another atropine injection.

Similarly the vasopressor effect of acetylcholine was preserved after clamping the abdominal aorta at the level of the diaphragm in one cat and two dogs and remained undiminished for two hours or more until the animals began to fail.

D. *The action of cocaine on the vasopressor response to acetylcholine.* Cocaine hydrochloride, if administered to animals in doses of 3.0 mgm. intravenously or 5 to 10 mgm. intramuscularly, produced a gradual rise in blood pressure with occasional cardiac irregularities. If the above doses of cocaine were administered to animals already under the full effects of atropine, subsequent injections of 0.025 to 0.15 mgm. of acetylcholine did not produce pressor effects. In several cases, it was observed that doses of acetylcholine producing no hemodynamic effects before cocaine produced depression of blood pressure following cocaine. This latter effect may be attributed to the cardiac and probably vascular toxic effects of cocaine.

In all experiments when vasopressor effects from 0.025 to 0.15 mgm. of acetylcholine were established by previous administration of atropine and physostigmine or prostigmin, cocaine enhanced and prolonged these vasopressor responses (see fig. 3). Occasionally these potentiated pressor effects were preceded by falls of blood pressure. Cocaine in a few experiments potentiated the pressor effects of both muscarine and doryl.

E. *Locus and mode of the vasopressor action of acetylcholine.* Acetylcholine pressor effects are similar in appearance and duration to those obtained with epinephrine or nicotine. This epinephrine-like action may be due to central effects, carotid sinus reflexes, mobilization of epinephrine from the suprarenal glands or of other pressor substances, e.g., from the liver, or to stimulation of any part of the sympathetic neurone by acetylcholine directly, or to a combination of these factors. To determine the part these factors may play in the elicitation of the acetylcholine pressor effect, the following group of experiments was performed.

1. *Carotid sinus and central effects.* In seven cats and nine dogs both carotid sinuses were removed, both superior cervical ganglia resected and the vagi cut above the ganglia nodosa. In these animals with blood pressures of about 150 mm. of mercury and treated previously with 5 mgm. of atropine and 1 mgm. of physostigmine, 0.1 mgm. of acetylcholine produced blood pressure elevations of 60 mm. of mercury or more.

In two cats the entire cerebrum, cerebellum, medulla and spinal cord were destroyed in an acute experiment, the animals being kept alive by artificial respiration and continuous intravenous infusion of 7 per cent gum

acacia solution in saline. In these two animals previously treated with 5 mgm. of atropine and 2 mgm. of physostigmine, 0.05 to 0.2 mgm. of acetylcholine produced rises up to 40 mm. of mercury which persisted after clamping the abdominal aorta. In these animals the blood pressure elevations were not accompanied by respiratory stimulation and movements of the animals.

2. *Removal of the adrenals or liver and evisceration by clamping the abdominal aorta.* The following experiments were performed in nembutalized animals treated with physostigmine (1 mgm.) and atropine (5 mgm.)

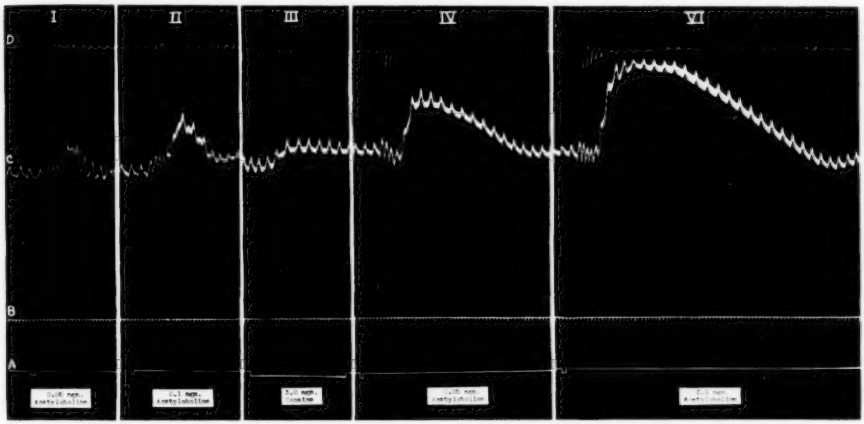

Fig. 3. Dog, 11.75 kgm., 30 mgm. of sodium pentobarbital, 5.0 mgm. of atropine sulfate and 1.0 mgm. of physostigmine salicylate per kilogram by vein.

A, line indicating injection of drugs. B, time, 2 seconds; base line representing zero mm. Hg pressure. C, blood pressure tracing from the common carotid artery. D, respiratory tracing from a tracheal cannula.

I, 0.05 mgm. of acetylcholine chloride per kilogram, vein. II, one minute later; 0.1 mgm. of acetylcholine chloride per kilogram, vein. III, two minutes later; 3.0 mgm. of cocaine hydrochloride per kilogram, vein. IV, two minutes later; 0.05 mgm. of acetylcholine chloride per kilogram, vein. V, one minute later; 0.1 mgm. of acetylcholine chloride per kilogram, vein.

and in most cases under artificial respiration, particularly after clamping of the aorta.

a. In ten dogs and five cats clamping the abdominal aorta at the level of the diaphragm did not prevent pressor effects from 0.05 mgm. of acetylcholine injected into the external jugular vein. The rises were sometimes smaller, but often as great or greater than those obtained previous to clamping of the aorta in spite of the higher blood pressure level. In both cats and dogs pressor effects from acetylcholine after clamping the abdominal aorta could be elicited as long as the animals lived.

b. In three dogs the entire liver was removed in acute experiments and the abdominal aorta clamped at the level of the diaphragm. In these animals 0.05 to 0.2 mgm. of acetylcholine produced blood pressure rises from 10 to 60 mm. of mercury.

c. In three cats and two dogs the skin of the anterior part of the animal was removed. Acetylcholine (0.05 mgm.) was then injected into the animals previously treated by atropine and physostigmine and the usual pressor effects obtained. Following clamping of the abdominal aorta, the same dose of acetylcholine usually produced a steep fall in blood pressure, whereas 0.1 cc. (total dose) of a 1:10,000 solution of epinephrine or 0.2 mgm. of nicotine produced rises of blood pressure. In one dog immediately after clamping the aorta acetylcholine produced pressor effects twice and further injections produced falls.

d. In fifteen animals both adrenals were removed and the blood pressure was kept above the shock level either by continuous intravenous infusion of 7 per cent acacia solution in saline or by clamping the abdominal aorta just below the diaphragm. In all these animals 0.05 to 0.1 mgm. of acetylcholine produced blood pressure elevations from 10 to 60 mm. of mercury. In other animals in which the adrenals were removed or the adrenal veins clamped and the resulting blood pressure fall was not compensated by either of the two above measures, acetylcholine produced a small or no rise in blood pressure. In one animal both adrenals were removed and the blood pressure rose to 90 mm. of mercury 1½ hours after the operation. In this animal 0.1 mgm. of acetylcholine produced a rise in blood pressure of 102 mm.

3. *The actions of certain autonomic drugs on the vasopressor response to acetylcholine.* Whereas physostigmine (section A) renders non-effective doses of acetylcholine effective and cocaine (section D) enhances the pressor effect of acetylcholine, nicotine (12) and ergotamine (13) reverse the vasopressor effects of acetylcholine in atropinized animals.

4. *The effect of acetylcholine in the absence of sympathetic ganglia.* In each of two dogs under nembutal anesthesia and artificial respiration both superior cervical and stellate ganglia and both thoracic sympathetic chains were removed. Following this operation the chest was closed and the animals allowed to breathe again. In these animals the injection of 0.05 mgm. of acetylcholine produced marked rises in blood pressure preceded by a small fall. Following this intravenous injection of acetylcholine the abdominal aorta was clamped at the level of the diaphragm and the experiments were continued using only the anterior portion of the animal which was completely deprived of its sympathetic ganglia. Injections of 0.05 mgm. of acetylcholine into the external jugular vein now produced without exception marked falls in blood pressure. In no instance was there the slightest evidence of a nicotinic action in the absence of the sympathetic ganglia (see fig. 4).

5. *Proof of liberation of pressor substances by acetylcholine.* In seven experiments two dogs were arranged so that blood from a donor receiving 5 mgm. of atropine and 1 mgm. of physostigmine could be rapidly transferred into a cocainized recipient (5 to 10 mgm. cocaine hydrochloride, intramuscularly). In each case the donor received 0.05 to 0.1 mgm. of acetylcholine; at the beginning of the pressor response 20 cc. of blood (in one animal 8 cc.) were taken and transferred into the recipient. This quantity of blood if injected immediately into the recipient produced pressor responses from $1\frac{1}{2}$ to 6 times the rise obtained from control blood injections. The same results were obtained in one experiment with cats in which 5 cc. of blood were transferred. In an experiment on dogs, in

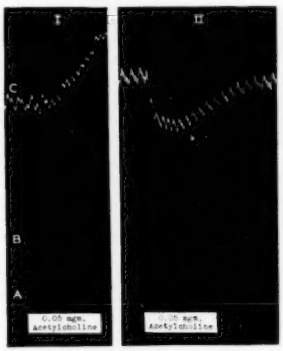

Fig. 4. Dog, 8.6 kgm., complete sympathetic ganglionectomy of the upper animal (both superior cervical ganglia and both thoracic chains removed), 30 mgm. of sodium pentobarbital, 3 mgm. of atropine sulfate and 1.0 mgm. of physostigmine salicylate per kilogram, vein.

A, line indicating injection of drugs. B, base line representing zero mm. Hg pressure. C, blood pressure tracing from the common carotid artery.

I, before clamping the abdominal aorta; 0.05 mgm. of acetylcholine chloride, femoral vein. II, after clamping the abdominal aorta at the diaphragm; 0.05 mgm. of acetylcholine chloride, external jugular vein.

which the abdominal aorta and adrenal glands of the donor were clamped before 20 cc. of blood were transferred, similar results were obtained. The results were negative in one dog following the transfer of blood after acetylcholine injection and in all cases in which the recipient was not cocainized. In three of the above experiments after 1 to 3 mgm. of nicotine were administered to the donor receiving atropine and physostigmine and transfer of blood was made following acetylcholine injection, no rise in blood pressure occurred in the recipient, whereas before nicotine administration to the donor definite pressor effects were obtained upon transfer of blood following acetylcholine injection into the donor.

In every case acetylcholine administered to the recipient produced

marked falls in blood pressure. Occasionally a rise in blood pressure followed by a secondary fall in the recipient occurred when transfer of blood was made following acetylcholine to the donor, particularly when the blood was taken too soon after acetylcholine injection or after a larger dose of acetylcholine. If the recipient received physostigmine in addition to cocaine, the transfer of blood following acetylcholine injection to the donor resulted in blood pressure falls in the recipient.

In seven dogs receiving amytal or nembutal anesthesia, it was found that the "apparent" blood epinephrine content ranged from 0.038 to 0.061 microgram per cubic centimeter as determined by Shaw's method (14) for the estimation of epinephrine. In these animals the "apparent" blood epinephrine concentration was not changed by the injection of 5 mgm. of atropine and 1 mgm. of physostigmine, the bloods being tested one-half to one hour after injection of these drugs. Blood taken at the beginning of the marked pressor responses to 0.05 to 0.1 mgm. of acetylcholine in these animals showed no alteration in the "apparent" blood epinephrine content. This indicates either that epinephrine is not liberated by acetylcholine injections or that the test is not sensitive enough to detect the increase. On the other hand, the "apparent" blood epinephrine content was increased 30 per cent in one dog (weight 6.8 kgm.) by the intravenous injection of a total dose of 0.25 cc. of 1:10,000 epinephrine solution, and in another dog (weight 7.3 kgm.) 334 per cent by a total dose of 0.25 cc. of 1:1000 epinephrine. The blood samples were taken from the femoral artery during an interval of 2 to 11 seconds after injection into the femoral vein on the opposite leg.

Discussion. The ablation of the carotid sinuses and the destruction of the entire central nervous system does not interfere with the acetylcholine pressor effect in atropine-physostigminized animals. It was further demonstrated that mobilization of epinephrine from the suprarenal glands and adrenergic substances from the liver or splanchnic viscera by acetylcholine were not singly responsible for the elicitation of the acetylcholine pressor response. Sympathomimetic substances which are liberated following acetylcholine injections produce definite physiological responses when transferred from one animal to another. This substance (or substances) produced through ganglionic stimulation by acetylcholine has many epinephrine-like actions. Chemical tests indicate that this sympathetic substance, or at least considerable portions of it, is not identical with epinephrine.

It is generally admitted that the splanchnic area and the skin contain most of the effectors on which adrenergic agents act in producing vasoconstriction. In the experiments in which the splanchnic viscera were excluded by clamping the abdominal aorta and the skin removed from the

anterior part of the animal, these effectors were excluded. According to expectations in these animals, though atropinized, acetylcholine produced only its inherent vasodilator effect.

The data reported in this paper establish the fact that the "nicotine-like" action of acetylcholine is due to stimulation of vasomotor sympathetic ganglionic cells. It was shown that nicotine abolishes and reverses the pressor effect of acetylcholine in intact atropinized or atropine-physostigminized animals, both before and after the clamping of the abdominal aorta. Similar reversals of the acetylcholine pressor effects were obtained upon clamping the abdominal aorta in animals in which the cervical and thoracic sympathetic ganglia were removed. This method of complete sympathetic gangliectomy is of interest because it can be carried out fairly rapidly in acute experiments and because it is a feasible method of removing or excluding all sympathetic ganglia with abolition of all "nicotine-like" actions of acetylcholine. Bacq (15) reported that he removed all sympathetic ganglia or sectioned all sympathetic postganglionic fibers and claimed that in atropinized animals following sympathectomy the intravenous injection of 5 mgm. of acetylcholine did not produce a contraction of the muscle of the nictitating membrane. Feldberg and Minz (9) attempted partial sympathetic gangliectomies in cats and found that removal of the stellate ganglia diminished the cardio-accelerator action of acetylcholine and that the removal of the solar plexus and of the inferior mesenteric ganglia diminished the splanchnic vasoconstriction produced by acetylcholine. These authors, however, admitted that they were unable to perform complete sympathetic gangliectomies and to abolish or reverse the pressor action of acetylcholine.

The threshold of the sympathetic ganglia to acetylcholine is much higher than that of the vasodilator receptors and in most animals at least 0.5 mgm. of acetylcholine is necessary to elicit the "nicotine-like" effects. It was found that the amount of the acetylcholine sufficient to stimulate the ganglia was considerably lowered by the administration of physostigmine or prostigmin. The fact that physostigmine potentiates markedly the pressor effects of acetylcholine, but not those of muscarine and doryl, indicates that at least in part the physostigmine potentiation of the acetylcholine pressor effect is probably due to its anti-cholinesterase activity.

Atropine interferes only with the "muscarine-like" actions of acetylcholine but its opposition to these latter effects is a relative and variable one. For example, large doses of acetylcholine may produce in the presence of small or moderate amounts of atropine a rise in blood pressure, whereas a smaller dose of acetylcholine may produce a fall. The small dose of acetylcholine does not reach the threshold of the vasomotor

sympathetic ganglia, while with the large doses the resultant pressor response is a mere algebraic addition of the nicotinic and muscarinic actions of acetylcholine.

Cocaine alone does not initiate vasopressor responses to small ineffective amounts of acetylcholine in the presence of atropine, but potentiates such responses only if acetylcholine is given in doses sufficient to produce small pressor effects. These experiments show that cocaine potentiates the pressor effect of substances liberated by acetylcholine stimulation of the sympathetic ganglia.

SUMMARY

1. The anti-cholinesterases, physostigmine and prostigmin, potentiate the pressor effect of acetylcholine but not that of muscarine and doryl.

2. Small doses of acetylcholine in the presence of physostigmine or prostigmin also produce acceleration of the heart, relaxation of the gut, stimulation of the ocular sympathetics, incoördinated somatic motor efforts and stimulation of respiration in atropinized animals.

3. The pressor effects produced by acetylcholine are preserved following removal of the carotid sinuses, the central nervous system, the adrenal glands, the liver, and/or clamping of the abdominal aorta at the level of the diaphragm. Upon the removal of the thoracic sympathetic chain and the superior cervical ganglia, acetylcholine does not produce pressor effects or other nicotinic actions following clamping of the abdominal aorta. Thus the site of the pressor effect of acetylcholine has been definitely localized in the sympathetic ganglia. Purely pharmacological evidence supports this conclusion (nicotine, a ganglionic paralysant, abolishes and often reverses the pressor effect of acetylcholine).

4. The substance or substances liberated by acetylcholine stimulation of the sympathetic ganglia may be transferred from one animal to another producing pressor effects in the recipient.

5. Cocaine does not initiate pressor responses from small ineffective doses of acetylcholine in atropinized animals, but it enhances these effects from effective doses of acetylcholine.

REFERENCES

(1) Hunt and Taveau. Brit. Med. J. **2:** 1788, 1906a.
Hunt. This Journal **45:** 197, 1918.
(2) Schmiedeberg and Koppe. Das Muscarin, Leipzig, 1869.
(3) Boehm. Arch. f. exper. Path. u. Pharmakol. **19:** 87, 1885.
(4) Mott and Halliburton. Proc. Roy. Soc. **141:** 211, 1899.
(5) Müller. Pflüger's Arch. **134:** 289, 1910.
(6) Hunt and Taveau. Hygienic Lab. Bull. **73:** 1911.
(7) Dale. J. Pharmacol. and Exper. Therap. **6:** 147, 1914.
(8) Simonart. J. Pharmacol. and Exper. Therap. **46:** 157, 1932.

(9) FELDBERG AND MINZ. Arch. f. exper. Path. u. Pharmakol. **163:** 66, 1931; **165:** 261, 1932.
(10) FELDBERG AND GADDUM. J. Physiol. **81:** 305, 1934.
(11) KOPPANYI, DILLE AND LINEGAR. J. Pharmacol. and Exper. Therap. **58:** 105, 1936.
(12) LINEGAR, HERWICK AND KOPPANYI. J. Pharmacol. and Exper. Therap. **65:** 191, 1939.
(13) LINEGAR. This Journal **129:** 53, 1940.
(14) SHAW. Biochem. J. **32:** 19, 1938.
(15) BACQ. Arch. Int. de Physiol. **44:** 112, 1936.
(16) KOPPANYI AND LINEGAR. Science **90:** 141, 1939a.
Science **91:** 297, 1940b.

ADAPTATION TO ESTROGEN OVERDOSAGE

An Acquired Hormone Resistance Without Antihormone Formation

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The fact that an animal may become resistant to the action of a hormone after a period of pretreatment has first clearly been shown for the parathyroid hormone (1). Later we made similar observations concerning the gonadotropic principles and concluded that in the latter case the resistance is due to the formation of hormone antagonistic substances since we found that "the blood of animals which have become insensitive to the anterior pituitary-like hormone of pregnancy urine is able to inactivate this hormone" (2, 3). On the basis of these experiments, Collip (4) formulated the theory that resistance to gonadotropic hormone develops because the organism produces a specific "antihormone" against this principle. Numerous other instances in which the antihormone theory of hormone resistance proved applicable have recently been considered in our detailed review of this subject (5). It was observed, however, that in many cases, hormone resistance may develop without the formation of demonstrable antihormones. Thus we consistently failed to reveal any parathyroid hormone neutralizing effect in the blood of animals pretreated with this principle (6). Similarly we showed that although chronic treatment with adrenalin fails to elicit the formation of a specific adrenalin antagonizing principle, it gives the organism a very marked resistance against this hormone (7, 8). We noted, furthermore that the enlargement of the pituitary, the adrenal cortex and the corpora lutea elicited in the rat by estrogen overdosage regresses after several weeks of treatment in spite of daily estrogen administration. We had to conclude therefore, that the organism may acquire estrogen resistance (9) although others have shown (10–12) and we may confirm (6) that estrin does not lead to antihormone formation. More recently we noted that young rats chronically treated with large doses of estrogens show a marked decrease in body weight during the first week but then reveal the development of resistance by the resumption of normal growth and weight increase. This readily distinguishable external sign of adaptation to the systemic effects of estrogen overdosage proved to be a useful indicator which served

as a basis for the experiments to be reported in this communication. The main purpose of these experiments was to analyze further the phenomenon of hormone resistance without antihormone formation and to establish the degree of specificity of such a resistance. As will be seen from the experiments reported in the experimental part of this paper, this resistance is not very specific inasmuch as pretreatment with estradiol renders animals resistant to the artificial synthetic estrogen diethylstilbestrol and *vice versa*.

EXPERIMENTAL PART. In our first experiment, we used 32 male and 32 female growing albino rats. At the beginning of the experiment the body weight of the males varied between 110 and 143 grams and that of the females between 107 and 145 grams. Eight males and 8 females received daily doses of 2 mgm. of estradiol subcutaneously in 0.1 cc. of peanut oil while 8 males and 8 females were given subcutaneous injections of the same

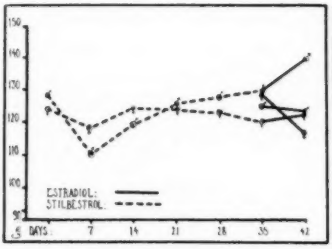

Fig. 1

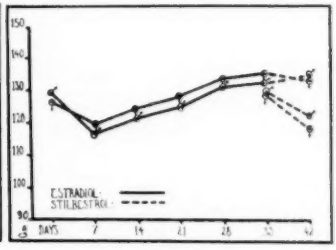

Fig. 2

Fig. 1. Weight curves of rats adapted to a large dose of stilbestrol, then treated with estradiol and of controls not adapted receiving estradiol during the last week of the experiment.

Fig. 2. Weight curves of rats adapted to a large dose of estradiol, then treated with stilbestrol and of controls not adapted receiving stilbestrol during the last week of the experiment.

daily dose of 4:4′-dihydroxy-α:β-diethylstilbene (diethylstilbestrol or stilbestrol) dissolved in the same amount of peanut oil. The animals were weighed at the onset of the experiment and then once a week throughout the treatment period. From their average weight, we constructed the growth curves shown in figures 1 and 2. These graphs indicate that during the first week, the rats of both sexes lost a great deal of weight as a result of the estrin treatment. By the end of the second week their weight had begun to increase, however, and in spite of continued daily treatment with the estrogens, the animals in all groups except in that of the diethylstilbestrol treated females continued to gain until the fifth week. In the latter group, after the recovery from the initial weight loss, the body weight remained fairly constant. It is noteworthy that although before treatment, the males of both experimental series were larger than

the females, the former lost so much more weight during the initial phase of estrogen administration that by the end of the first week, their average weight was below that of the females. At the end of the fifth week, the treatment of the animals adapted to estradiol was changed to diethylstilbestrol, while the animals which had so far been treated with the latter compound were given the same amount of estradiol for one week. As the graph indicates, this change in the type of the estrogen administered did not lead to any significant difference in the growth rate. At the same time, that is to say, during the fifth week of the experiment two other groups each consisting of 8 male and 8 female rats—which had not been pretreated but were approximately comparable in size to the estrogen pretreated animals—were given similar daily injections of estradiol and diethylstilbestrol respectively. The graph shows the considerable loss of weight which resulted from this treatment, a loss of weight which is approximately similar to that occasioned by the estrogens in the pretreated group during the first week of treatment. This experiment indicates quite clearly that resistance may be acquired to the toxic actions of both estradiol and diethylstilbestrol and that pretreatment with one of these estrogens renders the animals resistant to both estrogenic compounds.

It appeared of interest to establish whether a similar resistance to the toxic effect of large doses of estrogen may also be obtained by pretreatment with smaller doses. In order to determine this and to obtain further evidence supporting the conception of "crossed resistance" between the naturally occurring and artificial estrogens, we performed the following experiments.

Sixteen male (body weight 100–129 grams) and 16 female rats (body weight 96–120 grams) were divided into two groups, each of which contained 8 males and 8 females. One group received daily injections of 300 gamma of diethylstilbestrol and the other 300 gamma of estradiol subcutaneously in 0.1 cc. of peanut oil daily. Figures 3 and 4 show that this dose was tolerated without any loss of weight by the diethylstilbestrol treated males and the estradiol treated females while the remaining two groups showed a slight initial decrease in the average body weight. However growth was soon resumed by all groups and at the end of the fourth week, the body weight was far above the initial value in all animals. At this time, the estradiol pretreated rats were changed to daily injections of 1 mgm. of diethylstilbestrol while the animals pretreated with the latter substance received 1 mgm. of estradiol. Both substances were administered subcutaneously in 0.1 cc. of peanut oil. As indicated by our graphs, the trend of the growth curves was not significantly altered by this change to a large dose of a different estrogen. At the end of the fourth week, that is to say, at the time when the change in treatment occurred, we

began injecting a group of 8 males and 8 females with similar 1 mgm. daily doses of diethylstilbestrol and another group of 8 males and 8 females with the same amount of estradiol. These animals which had not been pretreated with estrogens showed a considerable decrease in body weight as indicated in figures 3 and 4, thus demonstrating that the pretreated group has really acquired a definite estrogen insensitivity.

Discussion. Our experiments indicate that the organism may adapt itself at least to the toxic effects of both estradiol and stilbestrol. While this is merely a confirmation and extension of previous observations (9, 13–15), it is of particular interest that adaptation to the naturally occurring steroid hormone, estradiol, induces resistance to a stilbene derivative which has a different chemical structure. At least in this instance, it appears that acquired hormone resistance without antihormone

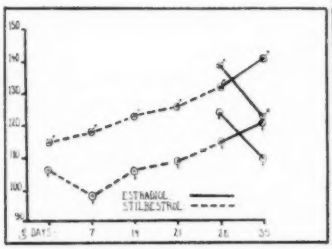

Fig. 3

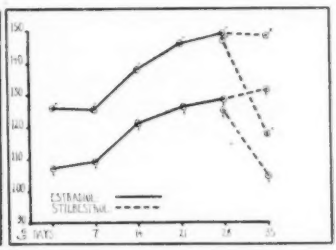

Fig. 4

Fig. 3. Weight curves of rats adapted to a relatively small dose of stilbestrol, then treated with a large dose of estradiol and of controls not adapted receiving estradiol during the last week of the experiment.

Fig. 4. Weight curves of rats adapted to a relatively small dose of estradiol, then treated with a large dose of stilbestrol and of controls not adapted receiving stilbestrol during the last week of the experiment.

formation is less specific than it usually is in cases of antihormone production. It will be recalled that resistance to gonadotropic preparations from the pituitary which is due to antihormone formation does not render animals insensitive to gonadotropic pregnancy urine extracts or *vice versa* (2, 3). Furthermore, antihormones elicited by treatment with a certain thyrotropic preparation have little or no effect on thyrotropic extracts prepared by a different method (16, 17).

With regard to the theoretical interpretation of the mechanism responsible for the crossed resistance between the two estrogens which we used, the following two possibilities present themselves. First it is conceivable that diethylstilbestrol is transformed in the organism to a substance similar to or identical in structure with estradiol and that it acts only after transformation into a compound of the steroid type. If this were

true, we would not be dealing with a real example of crossed resistance, since at the time when the estrogens which we employed exerted their pharmacological action, they would have become chemically identical or at least extremely similar. However, this interpretation receives no support from the bioassays which demonstrate that weight for weight, diethylstilbestrol is more active than most of the natural estrogens. The findings of Stroud (18) likewise indicate that the assumption of such a transformation is unfounded since he showed that diethylstilbestrol may be recovered as such from the urine of rabbits treated with this compound. It is true that the recovery is not quantitative but the author states that ". . . 4:4′-dihydroxy-α:β-diethylstilbene give recoveries of the order of 20 per cent compared with 1.5 per cent found for oestrone. This indicates a metabolic process for the synthetic oestrogens different from that of oestrone." These observations are not readily compatible with the view that diethylstilbestrol acts only after conversion into a steroid estrogen similar to the naturally occurring compounds.

A second and more likely explanation of our findings is that the organism does not become resistant to a certain chemical substance with estrogenic actions (that is to say, in our case to estradiol or diethylstilbestrol) as such, but to the estrogenic effect itself. If such an interpretation should prove to be correct, this case of crossed resistance would deserve special interest inasmuch as it would be a case of *adaptation to an action or effect rather than to a certain chemical substance or drug.*

Before closing this discussion, we should like to add that in numerous experimental series we noted that with very long continued estrone, estradiol or diethylstilbestrol treatment in doses of 2 to 5 mgm. daily, rats weighing approximately 100–150 grams at the onset of the experiment show the usual loss of weight during the first week followed by adaptation and increase in weight during the subsequent 5 to 6 weeks which is in accord with what has been said above. In these very chronic experiments however, where treatment was continued for 2 to 3 months, we invariably observed that after the period of adaptation, a third stage follows during which the acquired resistance appears to vanish so that the animals begin to lose weight again just as they did before adaptation occurred. We are not reporting these experiments in detail mainly because, unlike the initial period of weight loss and the period of adaptation, the final exhaustion of the adaptive mechanism and loss of weight is subject to great individual variations, one animal of a group beginning to lose weight after 5 or 6 weeks while another may go on for three months before its weight curve declines. Because of this great variability, each animal would have to be considered separately and since little would be gained from the study of such long individual weight curves or tables, we are omitting them here for the sake of brevity. Yet we want to mention these observations because they confirm our contention that the syndrome of adaptation to

estrogens is similar to that which develops during adaptation to various other damaging agents. It has been shown that during adaptation to almost any change in the internal or external environment of the organism, a so-called "general adaptation syndrome" is elicited which has three distinct stages. These have been termed the "stage of the alarm reaction," the "stage of resistance" and the "stage of exhaustion" respectively. During the first stage, among other symptoms and signs there is considerable loss of body weight, adrenal enlargement, involution of the lymphatic organs and water retention. During the second stage, most of these changes disappear and the animals become very resistant to the agent with which they are treated. Eventually however, during the third stage they again begin to lose weight and finally die with symptoms and signs similar to those seen during the initial stage. On the basis of these observations, we assumed that there is a general non-specific adaptation mechanism which facilitates the acquisition of resistance against a great many agents and that in case of prolonged exposure, this mechanism wears out and the adaptability or "adaptation energy" becomes exhausted (8). The literature on this adaptation syndrome has recently been reviewed by Leblond (18) and Varangot (19). Selye et al. (20) and Selye (21) showed that acute overdosage with estrogens may lead to a typical "alarm reaction" and the present experiments indicate that under the influence of continued treatment with estrogenic substances a "stage of resistance" and finally a "stage of exhaustion" develops. From this we may conclude that even adaptation to estrogens is not permanent but subject to the general law of the eventual exhaustion of adaptation energy as outlined in the above mentioned publications.

SUMMARY

Experiments indicate that the weight of growing rats chronically treated with estrogens first declines but growth is later resumed and the body weight may actually exceed the initial level in spite of continued administration of massive doses of estradiol or diethylstilbestrol. This is interpreted as additional evidence indicating that adaptation is possible at least to the toxic action of these estrogens although they do not elicit antihormone formation.

Rats adapted to the natural estrogen, estradiol, prove to be equally resistant to the artificial estrogenic compound, diethylstilbestrol, although chemically the latter is quite different from the former. Conversely diethylstilbestrol pretreatment renders the rat resistant to the toxic actions of estradiol. A review of the literature gives no support to the assumption that this "crossed resistance" should be regarded as due to the transformation of diethylstilbestrol into a naturally occurring estrogen similar to or identical with estradiol. It is concluded therefore, that the most probable interpretation of these findings is that we are dealing with a case of

acquired resistance to a certain (estrogenic) pharmacological action rather than to a particular chemical substance.

In case of very prolonged daily administration of relatively large doses of estrogens, the acquired adaptation is gradually lost. The animals, which following an initial stage of weight loss had adapted themselves to the treatment sufficiently to gain weight in spite of continued treatment, eventually lose weight again and finally die after two or three months of estrogen administration. It is concluded that adaptation to the toxic actions of estrogens takes place in the same manner as adaptation to most noxious agents, namely, by the development of the "general adaptation syndrome" with its characteristic three stages. *The eventual loss of an already acquired adaptation to estrogens gives further support to the conception that the adaptability or "adaptation energy" of the organism is a limited quantity and is gradually consumed while the organism puts up resistance against a stimulus to which it appears to be adapted.*

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REFERENCES

(1) Selye, H. Endocrinology **16:** 547, 1932.
(2) Selye, H., J. B. Collip and D. L. Thomson. Proc. Soc. exper. Biol. and Med. **31:** 487, 1934.
(3) Selye, H., J. B. Collip and D. L. Thomson. Proc. Soc. exper. Biol. and Med. **31:** 566, 1934.
(4) Collip, J. B. J. Mount Sinai Hosp. **1:** 28, 1934.
(5) Collip, J. B., H. Selye and D. L. Thomson. Biol. Reviews, **15:** 1, 1940.
(6) Selye, H. (unpublished)
(7) Selye, H. This Journal **122:** 347, 1938.
(8) Selye, H. This Journal **123:** 758, 1938.
(9) Selye, H., J. B. Collip and D. L. Thomson. Proc. Soc. exper. Biol. and Med. **32:** 1377, 1935.
(10) D'Amour, F. E., C. Dumont and R. G. Gustavson. Proc. Soc. exper. Biol. and Med. **32:** 192, 1934.
(11) Twombly, G. H. Endocrinology **20:** 311, 1936.
(12) Ssargin, K. D. and I. A. Pleteneva. Bull. Biol. e. Med. exper. USSR **3:** 455, 1937.
(13) Noble, R. L. J. Endocrinol. **1:** 128, 1939.
(14) Macdonald, I. G. Surgery **63:** 138, 1936.
(15) Emmens, C. W. J. Physiol. **95:** 379, 1939.
(16) Werner, S. C. Proc. Soc. exper. Biol. and Med. **34:** 392, 1936.
(17) Werner, S. C. Proc. Soc. exper. Biol. and Med. **34:** 390, 1936.
(18) Leblond, C. P. Ann. d'Endocrinol. **1:** 179, 1939.
(19) Varangot, J. La Presse Med. **48:** 103, 1940.
(20) Selye, H., C. M. Harlow und J. B. Collip. Endokrinol. **18:** 81, 1936.
(21) Selye, H. Fondation Singer-Polignac Sex Hormone Conference, Paris 1937.

THE ESSENTIAL NATURE OF PANTOTHENIC ACID AND ANOTHER ALKALI LABILE FACTOR IN THE NUTRITION OF THE DOG[1, 2, 3]

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In a previous publication (1) evidence was presented for the necessity of a factor separate from thiamin, riboflavin, nicotinic acid, and vitamin B_6 in the nutrition of the dog. This factor followed factor W in all fractionation procedures and we suggested the identity of these two factors. Since pantothenic acid, the chick antidermatitis factor, has been shown in several laboratories (2, 3, 4) to be essential for the rat, it seemed advisable to investigate its importance for the dog. The results obtained with the liver extract fractions reported previously were not entirely suited to a study of pantothenic acid, and it was decided to make a more comprehensive investigation of this problem. In this paper we wish to show that in addition to factor W, dogs require pantothenic acid and another alkali labile factor.

EXPERIMENTAL. Newly weaned puppies and older growing dogs were used in all of the experiments and the general experimental procedure was very similar to that employed in our earlier studies. Thiamin, riboflavin and nicotinic acid were supplied as previously described. In addition pure vitamin B_6 in aqueous-alcoholic solution was given orally twice weekly at a level of 60 micrograms per kilogram of body weight per day.[5]

The known chemical properties of pantothenic acid suggested two methods of removing it from growth promoting fractions of liver extract: 1, destruction with alkali, and 2, extraction with ether at acid pH. The assay method for pantothenic acid used in all of this work is that described

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by Snell et al. (5, 6). It has been the experience of this laboratory that this method of assay correlates very well with the method for the chick antidermatitis factor using chicks.

Alkali inactivation. It was found that heating the hexane-butanol extract (7) on the steam bath (87–93°C.) with KOH at a concentration of 0.2 N for two hours completely destroyed the pantothenic acid present. Since the hexane-butanol extract allowed good growth when supplemented with vitamin B_6, it was thought that the alkali inactivated extract might furnish a suitable vitamin B complex source free of pantothenic acid. For a source of pantothenic acid a concentrate prepared by Wilson Laboratories was used throughout the experiment.[6] This concentrate was prepared from liver extract according to the method of Woolley et al. (8). It contained 3.5 milligrams of pantothenic acid per cc. and on the solid

TABLE 1

Growth response of young puppies on pantothenic acid and pantothenic acid-free concentrates

DOG NUMBER	LIVER FRACTION	TEST PERIOD	INITIAL WEIGHT	TOTAL GROWTH	GROWTH PER DAY	REMARKS
		days	*kgm.*	*kgm.*	*kgm.*	
1	3% hexane butanol extract	42	2.45	2.125	0.051	Excellent growth
2	14.6 mgm. Wilson's pantothenic acid over assay period	18	2.125	0.375	0.021	Poor growth
2	10 mgm. Wilson's pantothenic acid + 3% alkali inact. hexane-butanol ext.	27	2.500	1.075	0.040	Good growth
3	4% alkali inact. hexane butanol ext.	16	2.70	0.725	0.045	Good growth but dog died suddenly

basis contained 3.5 per cent pantothenic acid. An ether extract of this concentrate at acid pH was also found to be suitable as a source of pantothenic acid, and this preparation was also used in some of the trials.

A litter of three puppies was then placed on experiment for a study of the response to these fractions. One received in addition to the four crystalline vitamins the original hexane-butanol extract at a level of 3 per cent; another received the alkali inactivated hexane-butanol extract at a level equivalent to 4 per cent; while the third received the Wilson Laboratories pantothenic acid concentrate at a level equivalent to about 500 micrograms of pantothenic acid per day. The response of these dogs is given in table 1. The positive control showed an excellent rate of

[6] All liver extracts used in this study were kindly furnished to us by Wilson Laboratories.

growth but the dog receiving only pantothenic acid grew very poorly. When the pantothenic acid preparation was supplemented with the alkali inactivated hexane-butanol extract a marked increase in the rate of growth resulted. The dog receiving only the alkali inactivated extract grew rapidly for 16 days and then died very suddenly. The autopsy revealed no evidence of infection of any kind nor any other obvious cause of death. The failure was undoubtedly nutritional.

To continue the study six older dogs were placed on the basal ration containing the four pure vitamins. All of these dogs showed a growth plateau followed by anorexia and a marked loss of weight. Definite growth responses resulted when the pantothenic acid concentrate or the acid ether extract of this concentrate was injected. If, however, the animal was maintained on this ration continuously after one or several responses to pantothenic acid, a point was always reached when no response could be elicited by pantothenic acid. This was also the case when the alkali inactivated hexane-butanol extract was included in the ration at a level of 3 per cent. Addition of whole liver extract or the alcohol ether precipitate fraction produced an immediate recovery, but the alcohol ether filtrate and high levels of the alkali treated hexane-butanol extract were ineffective. Typical results obtained with these dogs are shown in figure 1.

Ether extraction. The removal of pantothenic acid from liver extract was accomplished with the following procedure: 200 grams alcohol soluble liver extract no. 39796 was diluted to one liter with water and 1:1 H_2SO_4 added until the pH was approximately 2.0. The precipitate thus formed was filtered off and the filtrate was placed in a continuous liquid-to-liquid extractor and extracted with peroxide free ether for 5 to 7 days. The pantothenic acid content of this ether residue was found to be not greater than 14 micrograms per gram of original liver extract.

This fraction was then added to the basal ration at a level of 2 per cent. Several older growing dogs were used for this study, all of which had been on fractions supplying large amounts of pantothenic acid. On this fraction dog 7 gained almost 4 kgm. in 80 days, then a plateau in the rate of growth occurred followed by anorexia and diarrhea with a consequent loss of weight. At this point injection of 3.5 cc. of a pantothenic acid concentrate produced an immediate remission from this condition as shown in figure 1. The pantothenic acid concentrate was prepared from the alcohol ether filtrate fraction of liver extract powder followed by adsorption and elution of the vitamin from active norite as was employed in that concentrate prepared by the Wilson Laboratories. The eluate was finally extracted with ether at acid pH. This extract contained only 1.85 per cent solids of which 5.4 per cent was pantothenic acid. Several days after the peak of growth response was reached the animal was found in a deep coma and died a few hours later. The comatose state was characterized

by bradycardia, deep labored breathing, and a temperature of 105 degrees. This syndrome simulated in many respects that previously reported for dog 14 (1). Another dog on this residue for 88 days was found in a

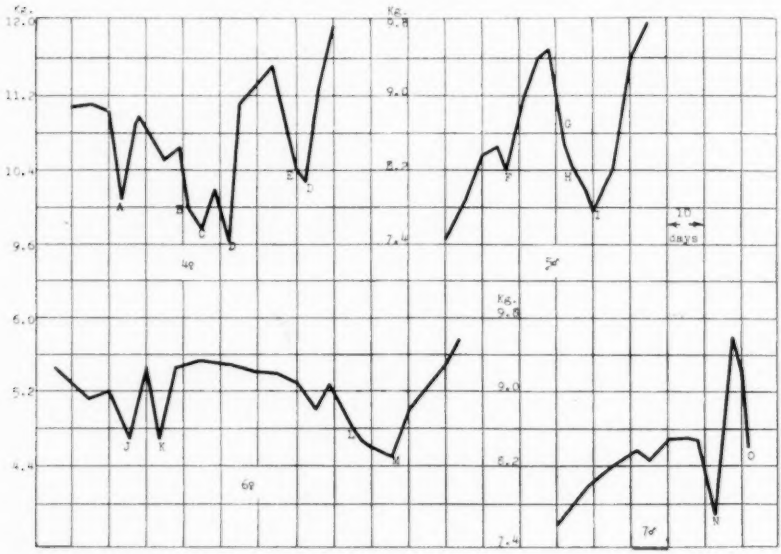

Fig. 1. Growth curves of older dogs on pantothenic acid deficient rations.

Dog 4—Basal ration. A, Wilson pantothenic acid concentrate equivalent to 20 mgm. pantothenic acid. B, Wilson pantothenic acid concentrate equivalent to 20.7 mgm. pantothenic acid. C, alkali treated hexane-butanol extract equivalent to 52 grams liver extract + 4 per cent on ration. D, 2 per cent alcohol ether precipitate on ration. E, Wilson pantothenic acid concentrate equivalent to 21 mgm. pantothenic acid.

Dog 5—Basal ration. F, Wilson pantothenic acid concentrate equivalent to 17.5 mgm. pantothenic acid. G, Wilson pantothenic acid concentrate equivalent to 45 mgm. pantothenic acid. H, alkali treated hexane-butanol extract equivalent to 80 grams liver extract + 5 per cent on ration. I, started 4 per cent liver extract powder on ration.

Dog 6—3 per cent alkali treated hexane-butanol extract on ration. J, Wilson pantothenic acid concentrate equivalent to 7.13 mgm. pantothenic acid. K, Wilson pantothenic acid concentrate equivalent to 14.0 mgm. pantothenic acid. L, Wilson pantothenic acid concentrate equivalent to 23.8 mgm. pantothenic acid. M, started 4 per cent liver extract 39796 on ration + 20 grams liver extract powder orally.

Dog 7—2 per cent acid ether residue. N, pantothenic acid concentrate equivalent to 8 mgm. pantothenic acid. O, animal died.

similar comatose condition and died shortly after. Autopsy of both dogs revealed mottled livers, severe gastritis particularly of the pylorus, and heavy bile pigmentation throughout the intestine.

To investigate further the use of ether residues in producing the deficiency a litter of six young puppies was placed on the basal ration supplemented with the four crystalline vitamins. Two were given the acid ether residue at a level of 2 per cent, two were given the acid ether extract at a level of 2 per cent, and the last two were given molasses at a level of 6 per cent. This molasses was found to be a good source of factor W accord-

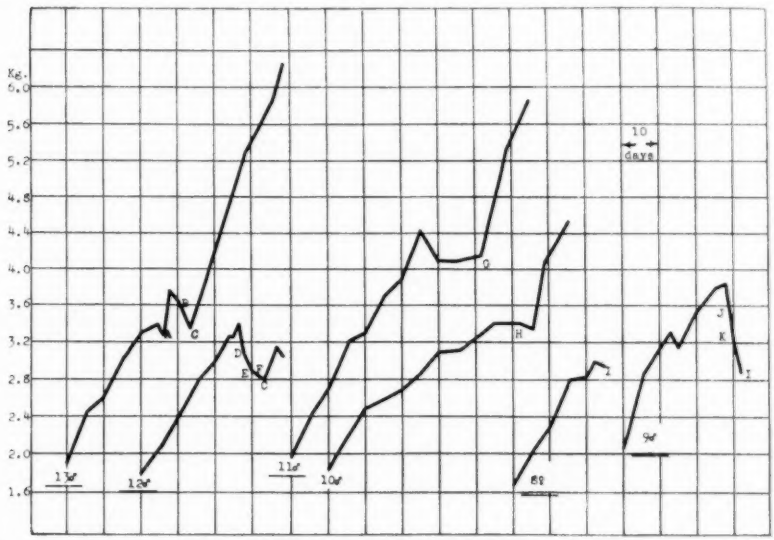

Fig. 2. Growth curves of young puppies on pantothenic acid and factor W deficient rations.

Dogs 12 and 13—6 per cent molasses. A, pantothenic acid concentrate equivalent to 1.89 mgm. pantothenic acid. B, acid ether extract equivalent to 25 grams liver extract. C, changed to 4 per cent liver extract powder. D, pantothenic acid concentrate equivalent to 3.8 mgm. pantothenic acid. E, 20 mgm. choline hydrochloride. F, acid ether extract equivalent to 22.5 gram liver extract.

Dogs 10 and 11—2 per cent acid ether extract. G, changed to 2 per cent alkali treated acid ether residue. H, changed to 2 per cent acid ether residue.

Dogs 8 and 9—2 per cent acid ether residue. I, animal died. J, pantothenic acid concentrate equivalent to 4 mgm. pantothenic acid. K, acid ether extract equivalent to 30 grams liver extract.

ing to the rat assay method and contained only 7 micrograms of pantothenic acid per gram. The growth response of these dogs is given in figure 2.

The two dogs receiving the acid ether residue grew very well for 4 weeks and then failed very suddenly. An injection of the pantothenic acid concentrate was given to dog 9 but no beneficial effects were observed. Upon

autopsy an intussusception was found in the lower small intestine which explains the anorexia and frequent vomiting movements as well as the primary cause of death. Light colored livers were found in both dogs. The yellowest was assayed for its riboflavin content by the method of Snell and Strong (9) and was found to contain 32 micrograms per gram of wet liver.

The two dogs receiving the acid ether extract grew fairly well but eventually plateaued after 7 weeks. Replacement of the extract with the acid ether residue or the alkali treated acid ether residue at a level of 2 per cent produced immediate responses in both cases. The latter fraction was prepared by diluting the acid ether residue 10 times with water, adding solid KOH to a concentration of 1 normal and then heating on the steam bath at 85–88°C. for $1\frac{1}{4}$ hours. The dogs receiving molasses grew very well for 4 weeks and then started to decline. Dog 12 failed to respond to pantothenic acid, choline, or the acid ether extract, but did show improvement for a time on liver extract powder until a serious infection incurred at the point of injection of the liver fractions. Dog 13 showed a slight response to the pantothenic acid concentrate, but not to a subsequent dose given as the acid ether extract fraction. The addition of 3 per cent liver extract powder to the basal ration resulted in an immediate recovery.

Discussion. It is apparent from the data presented above that the dog requires, in addition to factor W (the alkali stable factor), two essential factors which are destroyed by alkali. Thus dog 6 receiving the alkali treated hexane-butanol extract responded at first to injections of the pantothenic acid concentrate. Maintained on this fraction the animal became deficient again and would not respond to injections of much larger doses of pantothenic acid. Liver extract powder was required to restore his weight back to the original level. This was also true in the case of dog 5. After an initial response to pantothenic acid a relapse occurred which could not be cured by a combination of 45 mgm. of pantothenic acid in concentrate form and 80 grams of the alkali treated hexane-butanol extract given orally and parenterally as well as an additional 5 per cent of this fraction on the ration. Four per cent of liver extract powder supplemented to the basal ration completely restored the animal to normal.

The immediate temporary response of all dogs to injections of the highly purified pantothenic acid concentrates indicates strongly that this factor is required by the dog. The striking remission in the case of dog 7 was obtained on the pantothenic acid concentrate which represented only 17.5 grams of liver extract powder. It is unlikely that any factor other than pantothenic acid should follow so closely and so quantitatively. However, the matter cannot be established for certain until pure pantothenic acid is available.

The differentiation between pantothenic acid and the other essential

factors is further shown by the behavior of dog 4. Left on the basal ration alone the animal failed to respond a second time to 20 mgm. pantothenic acid and to large quantities of the alkali treated hexane-butanol extract. However, the addition of the alcohol ether precipitate at a 2 per cent level resulted in immediate recovery. This fraction contains no more than 20 micrograms of pantothenic acid per gram equivalent of liver extract powder. The recovery on such a fraction proves that there existed a deficiency of other factors.

Our experience with the acid ether fractions indicates that acid ether extraction removes other essential factors as well as pantothenic acid. The survival obtained with the acid ether extract alone at 2 per cent indicates that this fraction carries appreciable amounts of the alkali labile factor and just enough factor W for maintenance. The substitution of the alkali treated residue at a level of 2 per cent produced an excellent growth response further demonstrating the essential nature of the alkali stable factor W. The collapse of dog 7 could be ascribed to the inadequacy of the acid ether residue in factors other than pantothenic acid, yet the possibility of another acute pantothenic acid deficiency brought on by the rapid growth should also be considered.

The failure of dogs 12 and 13 to be maintained on molasses when supplemented with pantothenic acid indicates that molasses is also unsatisfactory as a B complex source for a study of pantothenic acid deficiency. Since molasses is adequate as a factor W source at 4 per cent for rat growth, it is probable that the dog requires still another factor. Recovery from this deficiency was obtained with 3 per cent liver extract in both cases although infection seriously hampered dog 12.

The complicated nature of the pantothenic acid deficiencies observed thus far reduces the significance of the variety of symptoms which we have seen. Irregular cardiac activity in tachycardia, less frequently in bradycardia, nausea and vomiting, intussusception, and finally loss of fur and dry scaliness of the skin suggest an impairment of autonomic control and especially that of the parasympathetic nervous system. What factor or factors these symptoms are attributable to cannot be seen in the light of present evidence.

SUMMARY

1. Evidence is presented for the necessity of pantothenic acid in addition to factor W in the nutrition of the dog. Dogs fail on alkali inactivated or acid ether extracted liver extract preparations and show a growth response upon the addition of concentrates of pantothenic acid. The rations employed thus far are not suitable for study of an uncomplicated pantothenic acid deficiency.

2. Dogs receiving the alkali treated factor W preparation and the

pantothenic acid concentrate do not show normal growth until other fractions are added to the ration. It is evident that there is an alkali labile factor other than pantothenic acid which is essential for the dog.

REFERENCES

(1) McKIBBIN, J. M., R. J. MADDEN, S. BLACK and C. A. ELVEHJEM. This Journal **128:** 102, 1939.
(2) HITCHINGS, G. H. AND Y. SUBBAROW. J. Nutrition **18:** 265, 1939.
(3) EL-SADR, M. M., H. G. HIND, T. F. MACRAE AND C. E. WORK. Nature **144:** 73, 1939.
(4) OLESON, J. J., D. W. WOOLLEY AND C. A. ELVEHJEM. Proc. Soc. Exper. Biol. and Med. **42:** 151, 1939.
(5) SNELL, E. E., F. M. STRONG AND W. H. PETERSON. Biochem. J. **31:** 1789, 1937.
(6) SNELL, E. E., F. M. STRONG AND W. H. PETERSON. J. Bact. **38:** 293, 1939.
(7) BLACK, S., D. V. FROST AND C. A. ELVEHJEM. J. Biol. Chem. **132:** 65, 1940.
(8) WOOLLEY, D. W., H. A. WAISMAN AND C. A. ELVEHJEM. J. Biol. Chem. **129:** 673, 1939.
(9) SNELL, E. E. AND F. M. STRONG. Ind. and Eng. Chem. (Anal. ed.) **11:** 346, 1939.

FACTORS AFFECTING THE MAINTENANCE OF COBALT POLYCYTHEMIA IN THE RAT[1]

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In the studies on cobalt polycythemia in this laboratory the observation has frequently been made that rats failed to maintain high levels of polycythemia. The level of hemoglobin and erythrocytes showed significant decreases, returning in some cases to normal. Subsequently the polycythemia would develop again, but it was difficult to maintain a high level of polycythemia for any length of time without these rather marked fluctuations.

The animals were receiving 0.03 mgm. of manganese per day which is considerably less than the level used by Orten et al. (1), but since these investigators have only reported studies on two relatively high levels (1.0 and 5.0 mgm. per day) no data were available which would indicate the level of manganese necessary for the stabilizing effect which they observed. The level of manganese which was employed in this study would be adequate for other known requirements hence other possible explanations for these effects were sought.

Since these animals were subjected to an abnormal hematopoietic stress it was possible that milk might not be sufficient in all respects as a basal food. Marshall (2) studying the effects of liver administration to polycythemic rats reported that liver extract produced a temporary depression in the polycythemia, followed by an increase to a level above the initially high level. Fresh liver produced an increase in the level of polycythemia without the temporary decrease noted with the extract. Considering these observations, then, it seemed possible that liver or certain liver fractions might aid in stabilizing the high levels of polycythemia, when added to the milk ration.

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[2] As a Commonwealth Fellow in this Department Doctor Underwood made the original observations which prompted this study. At present he is located in the Department of Agriculture in Perth, Western Australia.

In this report we wish to present the results obtained with various liver fractions as a supplement to the mineralized milk along with a modified procedure for drawing the blood samples for hemoglobin determinations.

EXPERIMENTAL. Male albino rats weighing approximately 200 to 250 grams were placed on a mineralized milk[3] ration. Hemoglobin determinations were followed as an index of polycythemia since the correlation between cell count and hemoglobin level has been well enough established (3) for the purposes of this experiment. Results of comparative determinations, however, on 12 of these animals showed that rats with a hemoglobin level of 23.2 grams per 100 cc. of blood had an erythrocyte count of approximately 11.6 million per cubic millimeter, as compared with the normal

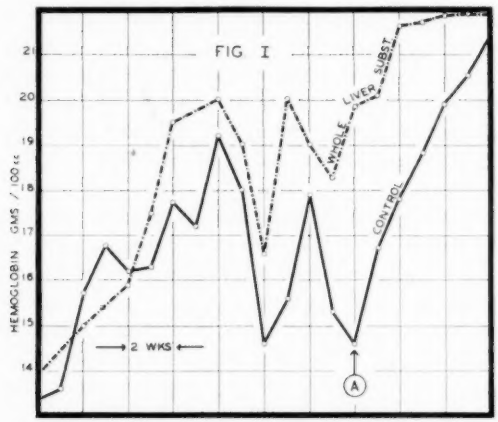

Fig. 1. Hemoglobin curves of rats receiving cobalt Note the consistently higher level of polycythemia in the rats receiving whole liver substance. *A* indicates the point at which the new method of drawing blood was employed.

hemoglobin level of approximately 14.5 and erythrocyte count of 7.5 million.

In the first series of animals, half of the group received 1 gram of Wilson's whole liver substance per day. Figure 1 summarizes the results obtained. Although each curve represents the average of three rats, the data are typical of many more. It will be noted that in both groups the fluctuations occurred in the hemoglobin curves although the animals on the liver supplement maintained a significantly higher level of hemoglobin.

Up to this time the technique employed for drawing blood samples had

[3] Daily supplement per rat included: 0.7 mgm. of Fe as the pyrophosphate, 0.07 mgm. of Cu as the sulfate, 0.03 mgm. of Mn as the chloride, 0.7 mgm. of Co as the chloride.

included clipping the end of rat's tail. The relative degree of injury to the animal became progressively worse as the experiment proceeded and it was difficult at times to prevent excess bleeding. Bleeding could be stopped by pressure on the tip of the tail or by heat cautery, but the animals would often reopen the wound when returned to their cages. Considerable loss of blood under such conditions prompted a search for a more satisfactory method for drawing the blood sample. Various methods were tried but the most satisfactory seemed to require the use of a razor blade or sharp scalpel with which the lateral vein of the tail could be severed, without cutting through the tail. The resulting clean cut would bleed freely but could be sealed readily by slight pressure of the forefinger directly over the cut. Employing this technique no more than 5 drops of blood were required from the animal to assure free flowing blood and an adequate sample for the hemoglobin determination. Likewise the inflammation which develops around the injured portion of the tail after clipping with scissors did not appear and the general disturbance of the animal was greatly decreased.

At the point *A* in figure 1 this modified technique was employed in the blood sampling. The resulting smooth curve indicates that hemorrhage may have had considerable influence upon the variability of the previous hemoglobin levels.

Since the animals receiving the whole liver substance showed a consistently higher level of hemoglobin than the control group, it appeared that liver also had a significant effect in maintaining the high level of polycythemia. In the next series then the effects of whole liver substance, liver extract and liver residue,[4] were studied.

Lowering the calcium-phosphorus ratio of a ration by adding NaH_2PO_4 has been shown (4) to increase iron utilization. It seemed possible that it might likewise speed up cobalt assimilation so in one group an amount of the NaH_2PO_4 was added to the milk sufficient to reduce the Ca/P to 0.6. Casein was employed as a supplement to another group to indicate the possibility of a purely protein effect. Lilly's P. A. concentrate for oral administration was employed with another group as a further possible fractionation of the liver substance.

Figure 2 summarizes the results obtained with those groups showing significant differences. Here again only typical data are presented, each curve representing the average of 3 rats. It will be noted that the liver extract (Lilly) allowed the highest level of polycythemia. In one case an animal attained a level of 28 grams of hemoglobin per 100 cc. of blood. Whole liver substance likewise allows a more rapid induction of polycy-

[4] These liver fractions were kindly supplied by Dr. David Klein of the Wilson Laboratories, Chicago, Illinois.

themia and the attainment of a higher level than the control animals. The NaH_2PO_4 practically eliminated the lag phase typical of the early part of the curve for the control group, but the animals exhibited the most marked symptoms of toxicity of the whole series. This observation is further substantiated by the depression of the growth curve. Of the growth curves in general it may be stated that the more rapid the production of polycythemia the more depressing the effect upon growth.

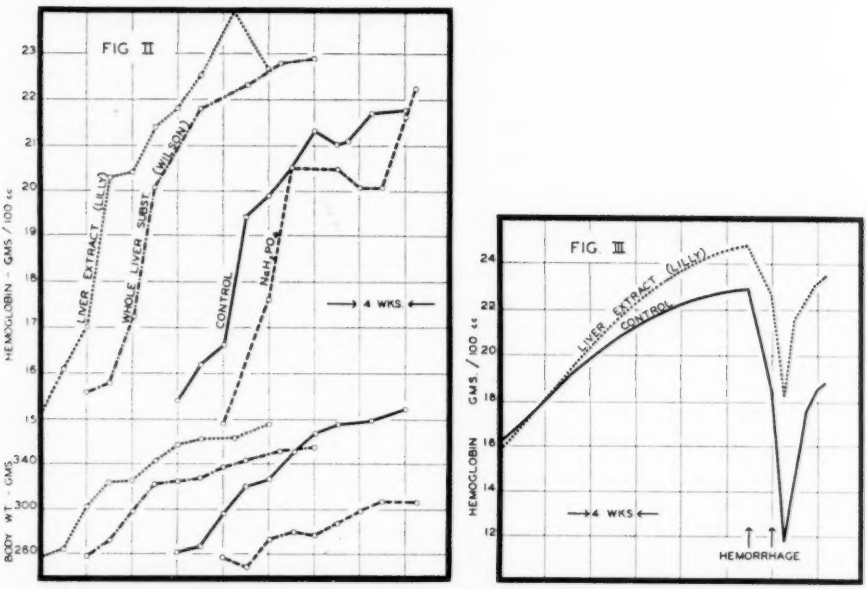

Fig. 2. Comparison of the groups of rats receiving supplements to the mineralized milk. Upper curves represent hemoglobin level, lower curves body weight. Note the higher levels of polycythemia in the animals receiving whole liver substance and liver extract. Note the rapid induction of polycythemia in the rats receiving NaH_2PO_4, with the resulting retarded growth.

Fig. 3. Hemoglobin curves of individual rats showing the effect of hemorrhage. Note the stabilizing effect of the liver extract.

The group receiving casein and the group receiving Wilson's liver extract showed only a slightly higher hemoglobin curve than the control group while the group on the liver residue showed a curve identical with the control group. Hence these curves were not included in this report.

It should be noted that the hemoglobin curves in this series are relatively smooth. Since the only difference between the groups of this series and those of the first series was the method of drawing the blood samples, it

appears that hemorrhage definitely affected the former results. As a further proof of the effect of hemorrhage, it was decided to produce a hemorrhage artificially, taking advantage of the tendency to bleed freely from a clean cut in the tail vein. In this case the tail was completely severed near the tip, and by frequently dilating the blood vessels with warm water and gently massaging the lateral veins toward the open wound, the animals could easily be bled to death. For our purposes, however, approximately 5 cc. were removed at a time and the results are shown in figure 3. The similarity between this curve and those obtained on the former animals in which hemorrhage was accidental, lends further evidence to the concept that the loss of blood was a significant factor in the failure to maintain high levels of polycythemia.

The supplemental effect of whole liver substance (Wilson) and liver extract (Lilly) was again demonstrated by the higher level of polycythemia produced, and by the fact that animals receiving the whole liver or liver extract failed to show the severe decreases in hemoglobin noted in the control group. In each case the control animal showed greater drops in the hemoglobin level although the amount of hemorrhage was equivalent.

Discussion. From the data presented in this report two facts seem evident. First, the technique for drawing blood samples cannot be the same as that generally used for young rats where only 3 or 4 determinations are required. The degree of injury and the resulting loss of blood introduce undesirable results into studies on the levels of blood constituents. The modified technique herein described has been used on rats of all ages and has proven to be highly satisfactory in all cases and definitely superior to the classical method of clipping off the tip of the tail for blood samples.

Second, liver and certain liver fractions have been shown to aid the rat in maintaining a high level of polycythemia when the animal is fed cobalt. These results are in agreement with the observation of Marshall (2) although our data are not directly comparable since in our study the animals received the supplements at the same time that cobalt administration began while Marshall fed the supplements after the polycythemia had been produced.

Our observations are interesting in light of the effect of choline hydrochloride or liver administration which has been reported by Davis (5) to depress cobalt polycythemia in dogs. The liver extract (Lilly) failed to depress polycythemia in the rats although it was fed at a level which would supply approximately 8 mgm. of choline per kilogram of body weight, the level found to be effective for dogs. The supplemental effect of certain liver fractions for the maintenance of polycythemia indicates that choline does not have a depressing action on the cobalt polycythemia of rats at the level fed.

Since we were able to maintain the control animals at high levels of polycythemia by modifying the bleeding technique, it would appear that the level of Mn employed was adequate, although the effects of higher levels were not studied.

SUMMARY

1. Fluctuations in the hemoglobin level of polycythemic rats have been largely prevented by modifying the technique of blood sampling to prevent unnecessary losses of blood.

2. Whole liver powder, liver extract (Wilson's) and a P. A. concentrate for oral use (Lilly) were found to aid the rats in producing and maintaining a higher level of cobalt polycythemia when added to a milk diet.

3. Casein had a very slight supplementary effect while liver residue (Wilson's) gave results identical with the control group.

4. When the calcium-phosphorus ratio of the milk was decreased to 0.6 by the addition of NaH_2PO_4 the rate of development of polycythemia increased with a resulting toxicity and depression of growth.

REFERENCES

(1) Orten, J. M., F. A. Underhill, E. R. Mugrage and R. C. Lewis. J. Biol. Chem. **99:** 465, 1933.

(2) Marshall, L. H. This Journal **114:** 194, 1935.

(3) Orten, J. M., F. A. Underhill, E. R. Mugrage and R. C. Lewis. Proc. Soc. Exper. Biol. and Med. **29:** 174, 1931.

(4) Anderson, H. D., K. B. McDonough and C. A. Elvehjem. J. Lab. and Clin. Med. **25:** 464, 1940.

(5) Davis, J. E. This Journal **127:** 322, 1939.

TYPICAL ANAPHYLAXIS IN THE DOG IN THE ABSENCE OF THE LIVER

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Anaphylaxis is characterized in certain species by the response of some particular tissues, leading to asphyxia in the guinea pig, heart failure in the rabbit and hepatic engorgement in the dog. These responses are, in general, regarded by students of anaphylaxis as the most obvious feature of a widespread tissue involvement. Even so, it has been generally accepted that anaphylaxis in the dog is not only characterized by liver involvement but does not occur in the absence of this organ. This view has been based on the experimental work of a number of investigators, who have interpreted their negative findings in this way. Also it has been supposed that any responses in extra-hepatic tissues are dependent upon the primary reaction in the liver and are the result of the liberation therefrom of toxic material into the circulation.

While there are differences of opinion on the mode of action of the liver in anaphylaxis in the dog, there has been almost complete agreement that this organ is essential for the production of typical canine anaphylaxis. Manwaring (1910) was unable to elicit anaphylactic shock in dogs with livers excluded by ligating various blood vessels. Voegtlin and Bernheim (1911) and Denecke (1914) excluded the liver by preparing Eck fistulas and clamping the hepatic arteries; subsequent injection of antigen gave no shock. Dale (1912) performed a few similar experiments and obtained no trace of shock with the liver excluded from the circulation, but on release of the clamp on the hepatic artery shock did develop. Weil (1917), demonstrating the marked congestion of the liver when antigen was injected into the portal vein or one of its branches, concluded that the congestion was a purely local phenomenon in the dog and was the only mechanism which takes part in the production of the vasomotor shock of anaphylaxis. Manwaring *et al.* (1925) found that their sensitized dogs, eviscerated or dehepatized by a modification of the Dale-Laidlaw Eck fistula technique, did not show the typical fall in blood pressure. When they mechanically obstructed the hepatic veins of a sensitized dog, Simonds and Brandes (1929) detected no further fall in arterial blood pressure when the antigen was injected, but it did occur on release of the obstruction. Again

these experiments were interpreted as further evidence that the liver was essential for the manifestation of canine anaphylaxis.

On the contrary, Pelz and Jackson (1918) claimed to have obtained anaphylactic shock after excluding the liver, but the single blood pressure tracing which they reproduce by no means represents the typical profound fall in blood pressure of the intact shocked dog. These experiments, in common with many another where exclusion of the liver was attempted by ligation procedures, may be criticized on the grounds that this organ was not completely isolated from the circulation. Thus Rich (1923) has shown by his experiments of injecting India ink into the jugular veins of dogs with ligatures around the portal vein, hepatic artery and inferior vena cava above the diaphragm, that some connection still exists by means of certain diaphragmatic vessels between the liver and the general circulation. Clearly it is preferable completely to remove the liver from the abdomen by surgical procedure. This we have done, and have obtained in these dehepatized sensitized dogs typical anaphylactic shock on injection of antigen. A preliminary account of these experiments has already been given (Waters, Markowitz and Jaques, 1938).

EXPERIMENTAL. The dogs were sensitized to horse serum which had been treated with alum. The serum was mixed with $\frac{1}{10}$th of its volume of a 10 per cent solution of potassium alum ($Al_2(SO_4)_3K_2SO_4\ 24H_2O$). This mixture was left in the refrigerator for 24 hours, then injected subcutaneously in amount equivalent to 0.5 cc. serum per kilo body weight. The shock dose of normal horse serum was given intravenously five or more weeks later. This method of sensitization we have found very satisfactory. The time required for sensitization appears to be longer than when untreated serum is given, but the animals remain highly sensitized for a much longer period. These results are in accord with the success obtained in sensitizing guinea pigs with ragweed pollen extracts treated with alum (Caulfield, Brown and Waters, 1935).

The hepatectomy operations were carried out under ether anesthesia by the one-stage technique described by Markowitz, Yater and Burrows (1933).

Dog 1. 7.2 kgm. Sensitization period: 8 weeks.

10:15 a.m. Ether anesthesia.
11:00 a.m. Liver removed from abdomen.
11:10 a.m. 20 cc. 25 per cent dextrose solution.
11:26 a.m. 20 cc. horse serum (jugular vein) injected over a period of 40 seconds.
11:55 a.m. Dog died.

Blood pressure (carotid) decreased evenly from 108 to 52 mm. Hg in 100 seconds of the beginning of the injection of serum. Further gradual fall until death.

Bladder response within 30 seconds of the beginning of serum injection.

Dog 2. 10.6 kgm. Sensitization period: 6 weeks.

11:30 a.m. Ether anesthesia.

12:15 p.m. Liver removed from abdomen.

12:30 p.m. 15 cc. 25 per cent dextrose solution.

12:50 p.m. 20 cc. horse serum (jugular vein).

15 cc. 25 per cent dextrose solution injected at ½ hour intervals.

2:37 p.m. Further injection of 20 cc. horse serum had no effect on the blood pressure (42 mm. Hg). Blood pressure decreased from 124 to 48 mm. Hg, sharply at first, then more gradually until 10 minutes after serum injection.

Bladder tone increased within 1½ minutes. Remained contracted for ¾ hr.

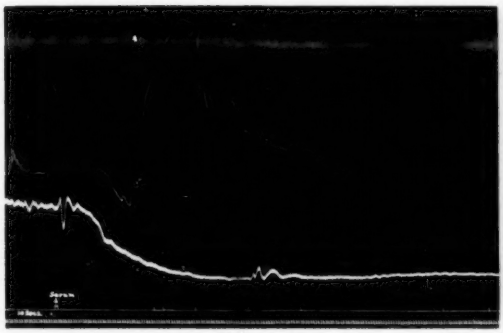

Fig. 1

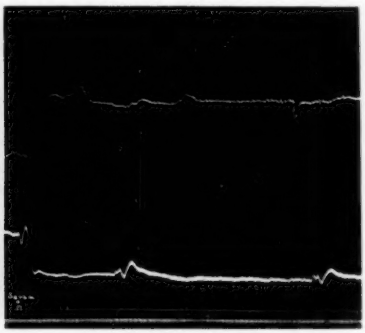

Fig. 2

Fig. 1. Liverless dog 3. Time intervals 10 seconds. Bladder (upper tracing) carotid blood pressure (lower). Injected with 1.3 cc. horse serum per kilo body weight. Slight elevation of blood pressure near center of tracing due to injection of dextrose solution.

Fig. 2. Liverless dog 4. Time intervals 10 seconds. Bladder (upper tracing) carotid blood pressure (lower). Injected with 0.5 cc. horse serum per kilo body weight. Two slight elevations of blood pressure due to injections of dextrose solution.

Four sensitized dogs, after fasting for 24 hours, were dehepatized under ether anesthesia, and while still anesthetized showed anaphylactic symptoms on injection of antigen. Details of two of these experiments are tabulated, and the kymograph records of the other two are reproduced in figures 1 and 2. Changes in bladder tone were obtained with a tambour connected to a cannula inserted through the bladder wall. Quantitative records of changes in pressure within the bladder were not made. Adequate dextrose was given to prevent hypoglycemia. The amounts of blood found in the abdominal cavity at post mortem were small.

Discussion. We have used as our criterion of shock the pronounced fall in arterial blood pressure which immediately follows the injection of the shock dose of antigen. This was first noted by Biedl and Kraus (1909) and has been amply confirmed by all who have tested it. We have given

some of our sera, in equivalent doses, to seven normal and to two eviscerated dogs under ether anesthesia, without any effect on the arterial blood pressure except in one of the normal animals which did show a fall in pressure of about 15 mm. Hg, but with complete recovery in about a minute. It is therefore evident that anaphylaxis occurred in these dogs in the complete absence of the liver, as judged by the most characteristic test, namely, the profound fall in blood pressure.

Our results appear to support the original conclusion of Biedl and Kraus (1909) that the decreased blood pressure is due to a primary peripheral vasodilatation, though it would be unwarranted to conclude that in the intact animal the hepatic changes may not be a contributing factor of some magnitude. Indeed, our tracings indicate that the fall in blood pressure takes place a little more gradually in the dehepatized than in the whole animal. But some of this difference may be due to the major operation which immediately preceded the injection of antigen. After noting the shock that developed in otherwise normal dogs into which had been transplanted livers from sensitized dogs, Manwaring, Hosepian and O'Neill (1925) considered it probable that extra hepatic factors are operative in acute canine anaphylactic shock.

Manwaring *et al.* (1925) were of the opinion that the tonic contraction of the bladder in canine anaphylaxis is due to the liberation from the liver of an anaphylatoxin with a histamine-like action. We have obtained the tonic contraction of the bladder in the absence of the liver. It has been clearly shown by several investigators (Dragstedt and Mead, 1936; Code, 1939) that histamine is liberated in canine anaphylaxis. It is difficult to decide whether the bladder response we obtained, in the absence of the liver, was due to a direct action of the antigen or to the production elsewhere of circulating histamine or similar substance. Dale and Laidlaw (1910) suggested that the increase in tone of the urinary bladder of the dog in histamine shock was due to anemia of the sacral portion of the spinal cord. Whatever the cause of the contraction of the bladder in anaphylactic shock the production of an "anaphylatoxin" by the liver is not essential, for contraction occurs in the absence of that organ.

SUMMARY

Typical anaphylactic shock has been obtained in sensitized dogs after complete surgical removal of the liver.

REFERENCES

BIEDL, A. AND R. KRAUS. Wien. klin. Wchnschr. **22:** 363, 1909.
CAULFIELD, A. H. W., M. H. BROWN AND E. T. WATERS. J. Allergy **7:** 451, 1936.
CODE, C. F. This Journal **127:** 78, 1939.
DALE, H. H. J. Pharmacol. and exper. Therap. **4:** 167, 1912.

DALE, H. H. AND P. P. LAIDLAW. J. Physiol. **41:** 318, 1910.
DENECKE, G. Ztschr. f. Immunitätsforsch. und exper. Therap. **20:** 507, 1914.
DRAGSTEDT, C. A. AND F. B. MEAD. This Journal **30:** 319, 1936.
MANWARING, W. H. Ztschr. f. Immunitätsforsch. und exper. Therap. **8:** 1, 1910–11.
MANWARING, W. H., V. M. HOSEPIAN, J. R. ENRIGHT AND D. F. PORTER. J. Immunol. **10:** 567, 1925.
MANWARING, W. H., V. W. HOSEPIAN, F. I. O'NEILL AND H. B. MOY. J. Immunol. **10:** 575, 1925.
MARKOWITZ, J., W. M. YATER AND W. H. BURROWS. J. Lab. Clin. Med. **18:** 1271, 1933.
PELZ, M. D. AND D. E. JACKSON. J. Lab. Clin. Med. **3:** 387, 1917–18.
RICH, A. R. Bull. Johns Hopk. Hosp. **34:** 321, 1923.
SIMONDS, J. P. AND W. W. BRANDES. J. Immunol. **13:** 1, 1927.
VOEGTLIN, C. AND B. M. BERNHEIM. J. Pharmacol. and exper. Therap. **2:** 507, 1911.
WATERS, E. T., J. MARKOWITZ AND L. B. JAQUES. Science **87:** 582, 1938.
WEIL, R. J. Immunol. **2:** 429, 1917.

INVOLUTION OF THE THYMUS OF THE YOUNG ALBINO RAT UNDER TREATMENT WITH TESTOSTERONE PROPIONATE

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It has been demonstrated that there is a distinct relationship between the thymus and the sex glands, in which the gonads exert an inhibitory effect. Using rats, Moore (1) showed that Antuitrin S stimulates hormone secretion by the testes, and Butcher and Persike (2) demonstrated that this increased hormone production arrests the growth of the thymus. This effect is through the gonads, since injections of Antuitrin S prove ineffective in arresting the growth of the thymi of castrated animals. On the basis of these observations, it seemed probable that testosterone propionate might induce regressive changes in the thymus, and this study was undertaken to determine the effect in young rats.[1]

METHODS. Throughout these experiments, only healthy litter-mate albino rats within 5 grams' body weight of each other were utilized, to avoid so far as possible the variations in thymic weights found in rats from different litters. No animals were autopsied at more than 100 days of age, at which time the thymus of the white rat generally begins involution (4). It has been demonstrated that the thymus reacts rapidly to disturbances in nutrition (5, 6, 7), so each group was kept in a separate cage, in which there was an excess of food and water at all times.

Each of these litter-mate groups consisted of several rats of the same sex, one serving as a control, the others being injected intraperitoneally with different amounts of testosterone propionate dissolved in sesame oil (0.1 mgm. to 1.0 cc.). There was an equal number of male and female groups. Usually, each rat was autopsied one day after the last injection. Body weights at the beginning of injections and at the time of autopsy were recorded. The relative weight of the thymus of each of the injected animals was compared to that of its litter-mate control, and the difference recorded in percent. The thymi of the majority of both experimental ani-

[1] After these experiments were started, Chiodi (3) reported that the thymi of white rats undergo atrophy under the influence of testosterone. Since the results of my experiments differ in certain respects from his, and since the data presented here are more complete than those obtained by him, it was deemed advisable to present this work.

mals and their controls were also examined for histological changes. Livers from most of the rats were examined in sections stained with hematoxylin and eosin and in frozen sections stained with Sudan III.

As approximately 150 litters, with a total of 357 rats, were used in these experiments, not all of the data obtained can be presented here. Typical litters, therefore, have been selected, and the data from these are presented in table 1.[2]

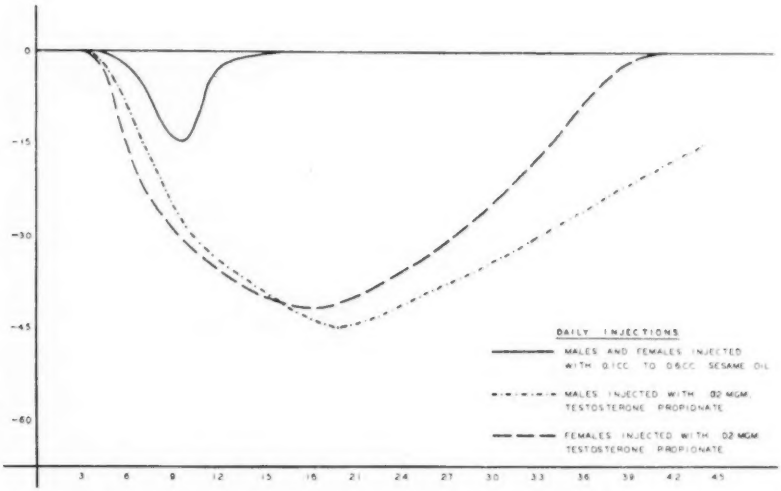

Fig. 1. The number of days of injections (x-axis) is plotted against the per cent of decrease (−) in the weight of the thymus of the injected rat as compared with the weight of the thymus of its litter-mate control (y-axis). The individual percentage points for each day of injections are averaged, and a smooth line graph is drawn from the average points thus obtained.

Observations. After 4 to 5 daily injections of 0.02 mgm. or more of testosterone propionate, the thymi of both male and female rats, between the ages of 20 days and 80 days, begin to involute. This involution reaches a peak at about the 19th day of injections. Figure 1 indicates that this decrease in size of the thymi of the injected animals is an involution and not an arrested growth.

The histological structure of the thymi of the injected rats changes slowly. By the 22nd day of injections, however, it is clearly evident that there are fewer thymocytes in both cortex and medulla, resulting in a less distinct differentiation between these two zones. The capsule becomes thickened,

[2] The complete data are on file in this laboratory, and will be made available to anyone who wishes to consult them.

TABLE 1

Representative protocols

SEX	GROUP NUMBER	NUMBER OF RATS	TREATMENT	NUMBER OF DAYS OF INJECTIONS	OBSERVATIONS AT AUTOPSY				
					Age	Body weight	Thymus weight	Per cent	
								+	−
						grams	*milligrams*		
♀	1	1	Control		81	74	220		
		1	0.01 mgm. daily from 61st day	20	81	72	178		16.9
♂	2	1	Control		80	58	145		
		1	0.01 mgm. daily from 62nd day	18	80	52	92		31.9
♀	3	2	Controls		74	76.5	218		
		1	0.02 mgm. daily from 55th day	18	74	72	127		37.7
		1	0.03 mgm. daily from 55th day	18	74	76	136		37.6
♂	4	1	Control		52	56	124		
		1	0.02 mgm. daily from 36th day	16	52	51	64		43.3
		1	0.03 mgm. daily from 36th day	16	52	58	77		40.4
♀	5	1	Control		62	111	213		
		1	0.02 mgm. every other day from 41st day	11	62	109	177		15.3
♂	6	1	Control		45	59	117		
		1	0.02 mgm. every other day from 28th day	9	45	56	79		28.8
♂	7	2	Controls		10	14	20.5		
		1	0.02 mgm. daily from 3rd day	7	10	12	15		11.8
		1	0.02 mgm. daily from 3rd day	7	10	8	10		14.5
♀	8	1	Control		6	9	13		
		1	0.02 mgm. daily from 3rd day	3	6	6	8		8.0
♀	9	1	Control		20	25	84		
		3	0.02 mgm. daily from 13th day	7	20	26	68		22.7

TABLE 1—*Concluded*

SEX	GROUP NUMBER	NUMBER OF RATS	TREATMENT	NUMBER OF DAYS OF INJECTIONS	OBSERVATIONS AT AUTOPSY				
					Age	Body weight	Thymus weight	Per cent	
								+	−
						grams	*milligrams*		
♀	10	1	Control		64	73	105		
		1	0.02 mgm. daily from 27–43rd days	17	64	79	152	33.3	
♂	11	1	Control			97	157		
		1	0.02 mgm. daily from about the 30th day for 16 days. Killed 21 days after last injection	16		110	193	8.4	
♀	12	1	Control			69	149		
		1	Castrated. 5 days later, 0.02 mgm. daily from about the 35th day	20		67	154	6.2	
♂	13	1	Control		69	110	216		
		1	Castrated. 4 days later, 0.03 mgm. from 58th day	11	69	110	212	2.0	
♂	14	1	Control			95	139		
		1	Castrated. 4 days later, 0.04 mgm. daily from about the 35th day	21		88	83		35.6
♂	15	1	Control			107	220		
		1	Castrated. Killed 21 days later			101	255	25.0	
		1	Castrated. 5 days later, 0.08 mgm. daily from about the 35th day	20		87	57		68.1

and there is an invasion and replacement of the normal thymic tissue by connective and adipose tissues.

Since the hormone was administered in oil, tests were made to determine the effect of the oil itself on the growth of the thymus. Thirty groups of animals were injected daily with sesame oil, in amounts ranging from 0.1 cc. to 0.6 cc. These injections did cause the thymus to involute slightly for a short time (fig. 1), the smallest injection producing about the same effect as the largest. By the 13th to 15th day of injections, however, refractoriness to the oil developed, and the weights of the thymi of the injected animals returned to normal (fig. 1).

To determine the smallest amount of testosterone propionate which would produce a maximum involution of the thymus of the average rat used, daily injections were varied from 0.01 mgm. to 0.04 mgm. Injections of 0.01 mgm. produced a moderate degree of involution (litters 1, 2: table 1). On the basis of data obtained from 24 groups, with a total of 76 rats, it would seem, however, that 0.02 mgm. was the optimal daily dosage, since it was as effective as daily injections of 0.03 mgm. and 0.04 mgm. (litters 3, 4).

Three groups of males were injected with this most efficient dosage (0.02 mgm.) every other day for a total period of 17 days. A like number of females was similarly treated for a total period of 21 days. While a certain degree of involution was obtained in all of the injected animals of these two groups, it was found that the percentage involution in these rats was not so great as that which occurred in those animals injected daily with the same dosage. (Compare litters 3, 4 with litters 5, 6.) For producing a maximum degree of thymic involution in the average rat used, the daily administration of 0.02 mgm. of testosterone propionate appears to be most efficient.

To determine the earliest age at which a change in the thymus could be induced, 10 groups of rats were injected daily with 0.02 mgm. of testosterone propionate from the second day of life, and were killed at various ages. In these very young rats, the effect of the injections was generally so great as to inhibit the body growth to such an extent that the injected animals usually weighed about one-fourth less than their controls, after only 6 to 7 days (litter 7). In these groups it was apparent that a decrease in the size of the thymus could be obtained at the 6th day of age (litter 8). It is a question, however, how early testosterone propionate itself will cause a change in the thymus, since the reduction in the size of the thymi of the rats in these groups may have been caused, not by the hormone, but by disturbances in nutrition, as is indicated by the failure of the injected animals to grow as much as their non-injected controls. On the other hand, rats injected with 0.02 mgm. of the hormone from the 13th to 19th day of life showed a very distinct involution of the thymus, accompanied by no disturbances in body growth (litter 9).

In gonadectomized animals injected with testosterone propionate, a regression in the size of the thymus was also obtained. Daily injections of 0.02 mgm. and 0.03 mgm., however, were not sufficient to definitely cause the thymus of the gonadectomized rat to involute (litters 12, 13) but injections of 0.04 mgm., 0.06 mgm. and 0.08 mgm. produced a definite reduction (litters 14, 15). Non-injected gonadectomized rats showed either unaffected or larger thymi than did their controls.

Certain rats were injected for long periods of time in order to determine whether or not refractoriness would develop. Females, injected daily

with 0.02 mgm. of testosterone propionate, exhibited refractoriness, since their thymi returned to normal size at about the 44th day of injections (fig. 1). Males, comparably treated, showed a certain degree of refractoriness by the 44th day, although this effect was not so pronounced or complete in the males as it was in the females (fig. 1). This sex difference appears to be statistically significant, since the probability against a difference of this magnitude occurring by chance is more than 1000:1.[3]

Eight groups of rats were injected with 0.02 mgm. of testosterone propionate for 17 days, and were autopsied at about 20 days after the last injection. The thymi of these rats had hypertrophied, and, in most cases, exceeded in weight the thymi of their litter-mate controls (litters 10, 11).

At autopsy, it was found that out of 139 injected animals, 28 had grown less, 18 had grown more, and 93 had grown neither more nor less than their controls. Since almost comparable numbers of the injected rats were either heavier or lighter than their controls, and since the great majority of the animals were unaffected at all by the injections, it would appear that daily administration of 0.02 mgm. of testosterone propionate was unable to bring about an effect on the growth or body weights of the injected animals, after the first 12 days of life.

Nevertheless, in spite of this lack of influence on growth rate, frozen sections stained with Sudan III., and fixed sections stained with hematoxylin and eosin from livers of rats injected with either sesame oil or testosterone propionate dissolved in oil showed marked fatty changes of the organs. In some cases, fatty infiltration occurred, particularly on the periphery of the livers, where they were in direct contact with the oil injected into the abdominal cavity. There was also some evidence of a mild chronic inflammatory reaction, probably as a result of the subjection of the hepatic tissue to a foreign substance.

The testes of the male rats, and the ovaries of the female rats which were injected with the hormone were smaller than those of their litter-mate controls. This inhibitory effect upon the gonads occurred gradually, but in the rats injected for longer periods of time, it was quite marked. The kidneys of rats injected with 0.02 mgm. of testosterone propionate generally were larger than those of their controls. This increase varied from 8 per cent to 25 per cent.

Discussion. The histological changes in the thymi which had undergone induced involution are similar to those occurring in normal involution, as described by Maximow and Bloom (8), and are much the same as those found by Butcher and Persike (2), Chiodi (3) and Smith (9).

The finding based on data obtained from many groups of rats that injections of sesame oil do have a depressive effect on the thymus for a short

[3] I am indebted to Prof. F. W. Weymouth for suggesting the statistical analysis and for checking the results.

time is not in accord with the report of Chiodi (3), who states that the decrease in the size of the thymus cannot be attributed to the trauma or toxicity of the oil vehicle in which the hormone was dissolved. As his animals were injected for 11 days with 0.5 mgm. of testosterone dissolved in 0.33 cc. of the same oil, and were killed 4 days after the last injection, I believe that some of the thymic involution obtained by him might have been directly due to the adverse effects of the oil in which the hormone was dissolved.

Chiodi (3) used two strengths of testosterone, 0.5 mgm. and 1.5 mgm., and thought that the degree of regression in the size of the thymus is related to the strength of the dosage. It would seem, though, that this inference is hardly justified, since many of our animals which were injected with only 0.02 mgm. of testosterone propionate daily showed as great a degree of thymic involution as did the relatively few used by Chiodi, which received so much more of the hormone for the same period of time—unless one is to infer that the difference in potency of the two preparations is in the ratio of about 75:1.

It is of more than passing interest that the thymi of those rats injected for 17 days and killed 20 days after the last injection hypertrophied rapidly after the injections were stopped. Butcher and Persike (2) also found this to be true in rats comparably treated with Antuitrin S. These results are in accord with those of Gottesman and Jaffe (10), who state that thymic remnants undergo hypertrophy after removal of the greater portion of the thymus. This suggests that there is some factor which maintains the thymus during immaturity. Chiodi (3) also found that the thymi of gonadectomized rats can be made to involute if the animals are treated with male sex hormone. That he failed to notice the difference in threshold is probably due to the fact that all of his dosages were excessive. Injections of testosterone propionate and the attendant organ changes had no appreciable effect on the body growth of the injected animals. Chiodi (3) and McEuen, Selye and Collip (11) also found no changes in body growth as the result of male hormone administration. Although this was generally true in the present series, very young animals were inhibited in their growth, as has been discussed above. Selye (12), using mice, likewise found that the kidneys of those animals receiving testosterone propionate were usually enlarged.

SUMMARY

The thymi of young albino rats of both sexes under 100 days of age were made to involute by injections of testosterone propionate, this involution reaching a peak at about the 19th day of injection. That this effect is significant is indicated by statistical analysis, which shows that the chances of such deviations as those observed being fortuitous are less than

1 in 1000. Daily administration of 0.02 mgm. or more caused the maximum degree of thymic involution in both sexes. This change, starting at about the 4th to 5th day of injection, was a real involution, not merely an arrest in growth. Refractoriness to prolonged injections of testosterone propionate occurred in both males and females, being more pronounced in females than in males. When the daily dosage was increased to 0.04 mgm. or more of testosterone propionate, an involution occurred in the thymi of gonadectomized animals of either sex. The thymi of both male and female rats hypertrophied when the injections were stopped. Injections of pure sesame oil caused a slight degree of thymic involution for a short time, after which refractoriness occurred. Testosterone propionate administration did not affect the body growth of the injected rats of either sex, except at early ages.

REFERENCES

(1) Moore, C. R. Am. J. Anat. **59:** 63, 1936.
(2) Butcher, E. O. and E. C. Persike, Jr. Endocrinology **23:** 501, 1938.
(3) Chiodi, H. Revista Soc. Argent. de Bio. **14:** 309, 1938.
Endocrinology **26:** 107, 1940.
(4) Donaldson, H. H. The rat. Wistar Institute, 1924.
(5) Capper, A. and R. A. Schless. J. Pediatrics **4:** 573, 1934.
(6) Hammar, J. A. Ztschr. f. mikr.-anat. Forschung **6:** 670, 1926.
(7) Hammar, J. A. Klin. Wchnschr. **8:** 2313, 1929.
(8) Maximow, A. A. and W. Bloom. A textbook of histology. W. B. Saunders Co., 1938.
(9) Smith, P. E. Anat. Rec. **47:** 119, 1930.
(10) Gottesman, J. M. and H. L. Jaffe. J. Exper. Med. **43:** 403, 1926.
(11) McEuen, C. S., H. Selye and J. B. Collip. Proc. Soc. Exper. Biol. and Med. **36:** 390, 1937.
(12) Selye, H. J. Urology **42:** 637, 1939.

ACTION POTENTIAL ARTIFACTS FROM SINGLE NERVE FIBERS

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The external, measurable action potential from a single element, the nerve fiber, has often been compared (cf. Gasser and Erlanger, 1927) to the potential drop across an external resistance R_e between the poles of a battery with internal resistance R_i. If the battery has an e.m.f., e, then

$$e = i(R_e + R_i), \tag{1}$$

where i is the current. Therefore in this analogy the external potential drop, the spike height, iR_e is determined by e, R_e, and R_i:

$$iR_e = e \frac{R_e}{R_e + R_i}.$$

e is the potential difference across the polarized membrane, R_e the longitudinal resistance outside the fiber, measured from the point where the crest of the impulse finds itself to the foot or the end of the spike, and R_i the corresponding resistance of the core of the fiber plus the resistance of the membrane at the electrically permeable region.

It is therefore to be expected that the size of the action potential can be modified by agents (fluids, metals, etc.) which change R_e. Any sudden change in the electrical conductance outside the fiber should abruptly change the action potential as it passes and theoretically should produce irregularities or artifacts in the recorded potential.

The purpose of the present paper is to investigate the effect of varying the electrical conductance external to single, isolated, unmyelinated fibers upon their electrical response and the potential field surrounding them during activity. The "artifacts" produced as a consequence of irregularities in conductance between the leads will be studied, and several of the more interesting ones will be analyzed.

A method of leading off with the fiber surrounded by a small quantity of solution was developed for these experiments. This method commends itself in general because there is little chance of damage to the fiber. It

is applicable to the study of other problems involving single fibers, particularly the action of pharmacological agents.

METHOD. Single nerve fibers were obtained mainly from the claw of the crayfish, Cambarus clarkii; they were prepared according to the method developed by van Harreveld and Wiersma (1936). For the most part the large motor axons of 20 to 40 μ diameter were isolated since these can be readily obtained free from connective tissue, which is not true of the smaller sensory fibers.

The prepared fibers, isolated for a distance of 20 to 25 mm., were either dissected from the claw with a bundle of nerve left attached at either end for ease in clamping, or else the proximal end was prepared in this way and the distal end left attached to the claw, the joints from which the fiber was prepared being discarded. The fiber was transferred to the leading off chamber, which had been filled with van Harreveld's (1936) solution, and the end, or ends, secured. If the fiber was left attached to the claw, the latter was held outside the end of the chamber, the nerve entering through a slot in such a way that it was kept covered with solution. Keeping the claw and therefore the muscle which the axon innervated attached to the preparation was done because the fiber maintained its activity much longer than one severed completely: 6 to 8 hours or more against one hour or less.

The leading off chamber employed in the experiments consisted of a triangular trough cut into a strip of Lucite which formed the bottom of a rectangular box of the same material; see figure 1, *A* and *B*. The trough had a vertex angle of 90°. Fluid leads entered at the vertex through fine pores of about 90μ diameter, spaced at intervals of 5 mm. in the case of the leading off electrodes. The other openings of the leads were through holes drilled into the side of the box and strip. Into these were inserted, ordinarily, platinum wires connected to the stimulator and amplifier. If the wires to the latter were of silver-silver chloride, nonpolarizable electrodes were obtained which were not poisonous to the preparation because of the length of fluid lead intervening. The free ends of the preparation were held in the vertex of the trough by overhead clamps, and one of these was movable by means of a lead screw.

The amount of fluid surrounding the fiber in the chamber could be limited by "lids" for the trough; see figure 1, *C*. These lids were made of a square rod of Lucite, beveled along one edge. This edge fitted into the vertex of the trough so that there a triangular cross-section of definite area was obtained.

After the fiber was in place along the bottom of the trough's V and the lid to be used was in place over the axon, the excess fluid was pipetted from the chamber, care being taken to blot up any fluid that could serve as a shunt around the leads. In this way the fiber was left surrounded

during the experiment by a limited quantity of fluid which formed the only appreciable conducting medium between the electrodes.

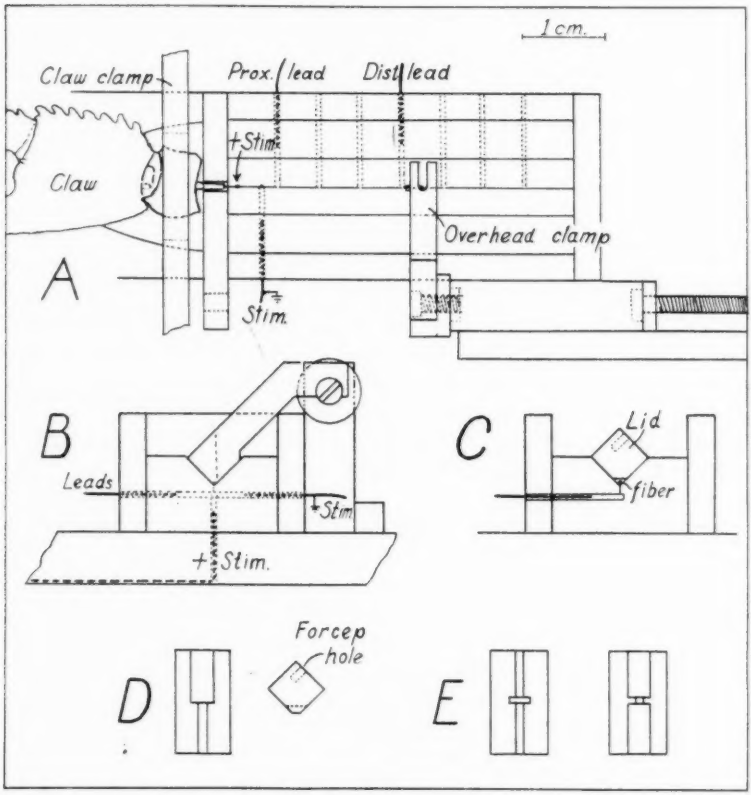

Fig. 1. Leading off chamber and lids.

A. Top view of chamber, showing trough, position of leads and preparation.

B. Side view of chamber from the stimulating, or proximal, end.

C. Cross-section of trough through a lead, with a lid and fiber in place.

D. Bevel view of lid used for producing a sudden change in conductance, and side view of this lid.

E. Bevel views of lids used for producing a sudden short increase and decrease in conductance, respectively.

The stimulator employed generated square waves whose frequency, duration and amplitude could be independently varied. The action potentials were led off through a push-pull differential amplifier into a cathode ray oscillograph. Very low grid-to-filament capacity tubes were

used in the first stages of amplification so that the current flow from the leads to ground and back through the preparation caused by the action potential would amount to less than 10^{-10} amps. Usually the stimulator and the sweep of the oscillograph were synchronized at a frequency of 30 per sec. This frequency did not produce signs of fatigue in the fiber, even after continuous stimulation for some minutes.

RESULTS. Before proceeding to a description of the artifacts produced by non-uniformity of conductance in the region between the leads, it will be necessary to consider the effect of the external conductance upon the action potential.

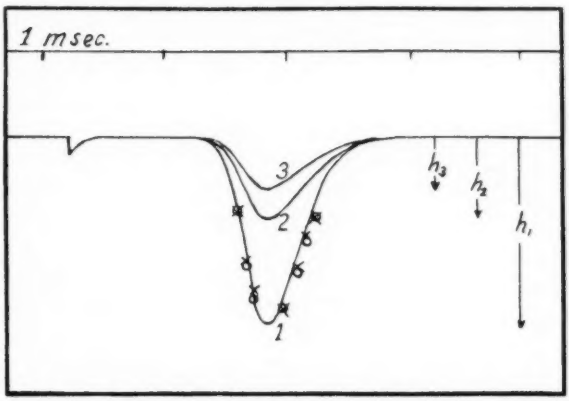

Fig. 2. Curves *1*, *2* and *3* are tracings of action potentials led off from the same fiber at the same distance (7 mm.) from the stimulating electrode; only the cross-section of the trough, and therefore the conductance, was changed. The crosses are the points obtained by multiplying ordinates of curve *2* by the ratio h_1/h_2 of the spike heights of curves *1* and *2*. Similarly, the circles are the point obtained by multiplying ordinates of curve *3* by h_1/h_3.

The maximum height of the action potential, with the smallest practicable amount of fluid, was about 1.5 mv. as compared to the 20 to 50 mv. which were obtained from these fibers when mounted in air or oil (cf. Hodgkin, 1938). As the amount of fluid in the trough was increased the height of the recorded spike declined. This change in height was the only one; the shape, duration and velocity of the action potential did not differ appreciably for different amounts of fluid when each amount was made uniform in cross-section by using a lid of constant bevel width. See figure 2 for a comparison of action potentials from the same fiber. That there was no difference in velocity such as Hodgkin (1939) found for single crab fibers immersed in sea water versus oil is explained by the fact that in the present case the external resistance, which is varied, is

small compared to the internal, and therefore in equation (1) the current i, which influences the velocity, will remain almost constant.

The question arises as to how much of the actual potential drop developed externally to the fiber and parallel to its axis is picked up by the electrodes under the different circumstances. To determine this point micro-electrodes were prepared, consisting of small glass tubes with one end drawn out into a capillary having a terminal inside diameter of approximately 10 μ. One of these electrodes was connected to one of the amplifier leads by means of a silver chloride-plated silver wire which dipped into the fluid contained in the tube. The other lead was by way of the trough in the usual manner. The capillary electrode was held by a micro-manipulator so that the pore of the electrode could be placed at any position relative to the fiber.

It was found that whether the pore rested on the surface of the fiber or was removed as far away as the fluid in the trough allowed (a range of 0.1 mm. in the case of least fluid up to 1 mm. or more with larger amounts) the height as well as the shape and duration of the action potential remained the same. If more solution was added the action potential decreased, but the decrease was the same irrespective of where the electrode was placed.

The result of this experiment implies that the equipotential lines describing the potential field external to the active region of the fiber extend out practically perpendicularly from the fiber's surface, and therefore the actual potential difference developed along the axon's external surface is led off. In other words, the medium about the nerve fiber acts as a linear conductor for the action *current*.

Artifacts produced by change in conductance. Bishop, Erlanger and Gasser (1926) discovered that distortions of the action potential record from myelinated nerve arose when idle leads, drops of water, undissected tissue, dead fibers, or cut branches occurred in the led-off region of the preparation. These were described as furnishing extra leads, as indeed they may. Schaefer and Schmitz (1934) showed that some of these types of artifacts were produced in myelinated nerve by variations in the external conductance along the nerve.

Early in the present work it was discovered that small variations in the medium surrounding the isolated fiber as well as damage to it gave rise to records riddled with irregularities. One type of injury common in unmyelinated fibers unless carefully handled is constriction which would not only abruptly change the external but also the internal resistance of the fiber at the narrowed place. These were the main reasons why the trough method was developed, for with this technique the fiber could be maintained in good condition and, if the trough and lid were made precisely enough, unwanted artifacts could be avoided.

Any variation in R_e (or R_i) as the action potential is conducted between the leading off electrodes will produce a spurious response in the record. This is caused by a change in the form of the action potential which will alter the potential of one lead relative to that of the other while the change is taking place (see diagram and analysis below). These artifacts can be especially well shown in single unmyelinated fiber preparations because the shortness of the wave-length of the impulse, about 5 mm., allows the artifact to be recorded distinct from the response at the first lead.

There are several types of spurious response, which can be produced under controlled conditions, that are of special interest: 1, those caused by a sudden increase or decrease in conductance, and 2, those produced by a sudden increase or decrease limited to a short distance ($\frac{1}{2}$ to 1 mm.) of the led-off stretch of fiber. The desired variations in conductance were accomplished by regulating the cross-section of solution in the trough with the aid of the special lids illustrated in figure 1, *D* and *E*. It will be shown below that the first type of artifact is proportional to the action potential itself, and that the second is proportional to the first differential of the action potential. Figure 3 illustrates examples of the two types.

In figure 4 A the situation in case (1) is shown diagrammatically. As the impulse travels from region 1, where the conductance per unit length measured along the fiber as C_1, to region 2, where the corresponding conductance decreases to C_2, the current flow outside of and along the fiber remains practically the same but the potential drop in region 2 is greater than it would have been in region 1 because of the decrease in conductance. Therefore lead G_2 will be at a higher potential than G_1. In figure 4 B the potential along the fiber is plotted against its length; the equipotential lines in A were drawn from the potential plot in B.

It is possible to derive a mathematical expression for the resulting recorded artifact. Let

$$E_1 = E_1(x - vt)$$

be the function of the parameters x and t, the distance along the fiber and the time respectively, which will represent the traveling action potential wave in a medium having the conductance, C_1 of region 1 of figure 4; v is the velocity of the wave. Let E_2 be a similar function for the wave in a medium having the conductance, C_2, of region 2. Now if h_1 and h_2 represent the spike height in regions 1 and 2, respectively, then, at least to a good approximation,

$$E_2 = \frac{h_2}{h_1} E_1(x - vt). \qquad (2)$$

This follows from the experimental fact that the shape, duration and velocity of the wave does not change under the present conditions (cf. fig. 2).

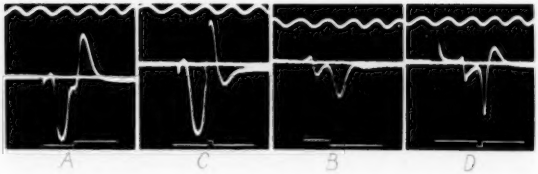

Fig. 3. Records of artifacts. In each case the change in conductance producing the artifact occurred at 6 mm. from the first lead. Therefore the first negative deflection is a record of the action potential itself, while the ensuing variations, the start of which is marked by an X, is the artifact. Under each record the change in conductance is shown diagrammatically. Monophasic leading off; time, 1 msec. See text for further references to records.

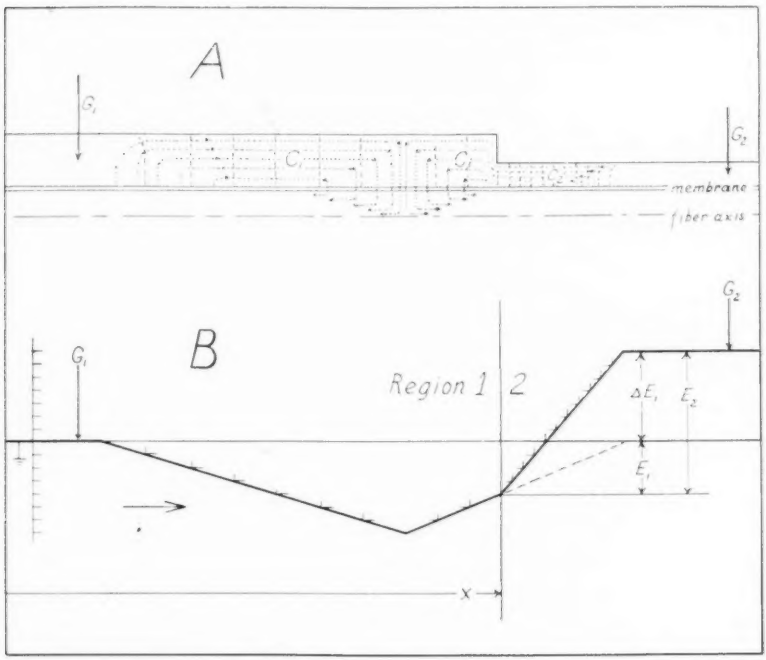

Fig. 4. A. Scheme of the current flow outside the fiber when the impulse encounters a sudden decrease in conductance. The equipotential lines are constructed from B, a diagram of the potential along the fiber. See text for analysis.

As a consequence of this fact it can be assumed that the current leaving or entering the membrane along the wave does not change as the external resistance is altered, and therefore from equation (2)

$$\frac{h_2}{h_1} = \frac{R_{e_2}}{R_{e_1}} = \frac{C_1}{C_2}$$

so that

$$E_2 = \frac{C_1}{C_2} E_1 . \tag{3}$$

Furthermore, since the external medium serves as a linear conductor, in the neighborhood of the discontinuity the part of the wave in region 1 will be represented by E_1 and the part in region 2 by E_2. Therefore

$$\Delta E_1 = E_2 - E_1, \text{ at } x,$$

where ΔE_1 is the increment of E_1 produced by the sudden change in conductance, as shown in figure 4 B. From equation (3), then,

$$\Delta E_1 = \frac{C_1 - C_2}{C_2} E_1 , \text{ at } x,$$

$$= G_1 - G_2 ,$$

where G_1 is the potential of the first lead relative to ground and G_2 that of the second. This means, of course, that the recorded artifact, expressed by the increment ΔE_2, is proportional to the action potential.

This artifact is of some use in certain types of experiment, for instance those with drugs, since effectively an extra lead can be obtained at any point in the led-off stretch by merely shifting the special lid. Examples of this type of artifact were shown in figure 3, A and B. Actually, the conditions diagrammed in figure 4 are those under which figure 3 B was taken; 3 A is the converse case.

Expressions for the artifact shown in C and D of the latter figure can also be readily derived. Referring to figure 5 A, the action potential in region 1 suddenly encounters a narrow region 2 of different conductance. Using the same notation, it will be seen that

$$\Delta E_1 = \delta E_2 - \delta E_1 .$$

Since

$$\delta E_2 = \frac{C_1}{C_2} \delta E_1 ,$$

$$\Delta E_1 = \frac{C_1 - C_2}{C_2} \delta E_1 .$$

If Δx is sufficiently small compared to the wavelength, then

$$\delta E_1 \overset{a}{=} \frac{\partial E_1}{\partial x} \Delta x, \text{ at } x.$$

Therefore,

$$G_1 - G_2 = \Delta E_1 \overset{a}{=} \frac{C_1 - C_2}{C_2} \frac{\partial E_1}{\partial x} \Delta x, \text{ at } x. \tag{4}$$

The recorded artifact is thus proportional to the first differential, $\frac{\partial E_1}{\partial x}$, of the action potential, which changes sign at the crest of the spike, accounting for the diphasic response seen in figure 3, C and D.

A spurious response very similar to the latter artifact will be recorded from a preparation where there is an abrupt but short increase in conductance, such as could be caused by an idle electrode. Of more interest,

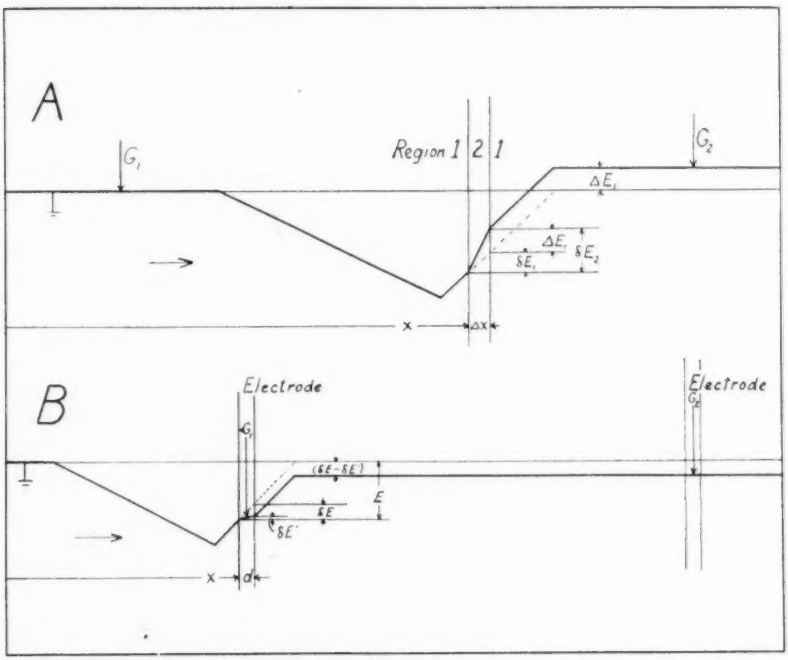

Fig. 5. A. Diagram of the potential along the fiber at an instant when the impulse is passing a sudden short decrease in conductance, the circumstance producing the artifact shown in figure 3 D. B. Similar potential diagram for the case where the impulse is passing an electrode of high conductance. See text for analyses.

however, is the question of the effect of the increased conductance furnished by the active leads. The last result, equation (4), will be of help in finding an answer.

In figure 5 B the impulse is traversing an active electrode covering the region x to $(x + d)$. If E is the function expressing the potential of the traveling wave in the region before and after the electrode, having the conductance C, and E' the function in a region having the conductance

C' of the electrode, then the potential, G_1, of the first electrode relative to ground can be expressed by

$$G_1 = E - \frac{\delta E'}{2}, \text{ at } x;$$

and the potential, G_2, of the second electrode by

$$G_2 = \delta E - \delta E' \overset{a}{=} \frac{C - C'}{C'} \frac{\partial E}{\partial x} d, \text{ at } x,$$

from equation (4), when d is small. When the electrode has a very high conductance so that $\frac{C - C'}{C'} \overset{a}{=} -1$, the difference in potential of the electrodes can be expressed by

$$G_1 - G_2 \overset{a}{=} E + \frac{\partial E}{\partial x} d, \text{ at } x,$$

and from Taylor's series

$$G_1 - G_2 \overset{a}{=} E\,(x + d - vt), \text{ at } x.$$

Therefore when the first electrode has low resistance it will not start to lead off the action potential until the impulse has traversed most of the width of the electrode. This conclusion agrees with experimental evidence obtained by Bishop, Erlanger and Gasser (1926) and others.

It can be concluded that great care must be exerted when leading off from single isolated fibers, where the environment of the fiber can be easily influenced, to avoid the presence of unwanted conductance artifacts. On the other hand, here such artifacts can be most readily recognized. This is not always the case in more complicated tissue such as whole nerve or the central nervous system. It is possible that some potential variations found in these tissues and ascribed to other causes are really due to irregularities in conductance.

SUMMARY

A new method for leading off the action potentials from single unmyelinated nerve fibers is described; the fiber is mounted in a Lucite trough and surrounded by a small amount of solution.

It is found under these conditions that though the external action potential decreases as the conductance about the fiber is increased, the shape, duration and velocity of the action potential remain unchanged within the limits of experimental error. It is shown that the medium about the action potential acts as a linear conductor for the action current.

Artifacts resulting from irregularities in the conductance outside the fiber are described. Two special types of artifacts, one giving a response proportional to the action potential and one a response proportional to its first differential, are analyzed.

REFERENCES

BISHOP, G. H., J. ERLANGER AND H. S. GASSER. This Journal **78:** 592, 1926.
GASSER, H. S. AND J. ERLANGER. This Journal **80:** 522, 1927.
HARREVELD, A. VAN. Proc. Soc. Exper. Biol. Med. **34:** 428, 1936.
HARREVELD, A. VAN AND C. A. G. WIERSMA. J. Physiol. **88:** 78, 1936.
HODGKIN, A. L. Proc. Roy. Soc. B, **126:** 87, 1938.
J. Physiol. **94:** 560, 1939.
SCHAEFER, H. AND W. SCHMITZ. Pflüger's Arch. **234:** 737, 1934.

SOME FACTORS WHICH INFLUENCE THE INHIBITION OF WATER LOSS IN ALBINO RATS BY PITUITRIN[1]

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Most experiments concerned with the effect of posterior pituitary extracts on body water have been performed upon mammals or frogs. In mammals the almost universal method of measurement of such effects has been the recording of changes in the volume of urine. In frogs, the usual reaction investigated is one which has been referred to as the Brunn reaction after Fritz Brunn who discovered the reaction in 1921 at Prague (3) and in this reaction an increase in total body water following the injection of posthypophyseal extracts is measured by changes in body weight. Superficially, the Brunn reaction and the mammalian "diuretic-antidiuretic" reaction appear to have little in common but a number of recent investigations in this laboratory (for example, 2) have brought to light evidence which indicates that in at least a number of respects they are fundamentally alike; apparent differences were found due to differences in the rate and nature of water exchange in the two classes of vertebrates.

In most previous investigations from this laboratory, the problem of phyletic variations between amphibians and mammals has been approached from the amphibian end. In the present study, we decided to approach it from the mammalian end. It seemed possible that the apparent differences between the Brunn and mammalian reactions might be due to differences in the method of measuring the effect of posterior pituitary extracts. Hence we decided to measure any effects not as changes in urine volume but as changes in the body weight of mammals. Using albino rats given water by mouth in the familiar water diuresis technique, we found that suitable doses of pituitrin would inhibit the loss of weight in such animals. To establish conditions necessary for the standardization of such a reaction, our first problem was to find the effect of various factors which might conceivably affect the results. We wish now to report on such factors as we have investigated to date.

Selected, mature, healthy albino rats were housed in rooms with partial

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temperature control and fed on Purina Fox Chow Checkers with iodized water to drink ad libitum, except for 15–18 hours previous to an experiment when no food or water were allowed. The general technique consisted in choosing some 15 to 20 rats from this colony, weighing them to the nearest 0.1 gram on a trip scale balance, placing them in individual, small cages, administering 5 per cent of their body weight of distilled water by mouth, injecting half of the number with pituitrin and subsequently re-weighing all animals every half-hour or hour. Mean percentage changes in weight were plotted against time.

Significance of control curves. The number of animals which may comprise a group is of necessity limited in any one man-day experiment. In Burn's method of assay of pituitary antidiuretic activity, each dose group consists of 4 rats (5). We have used a minimum of 6 rats to a group and the problem arose as to what variation might be expected between control groups of this number of rats. In other words, how far away should any 6-rat mean curve be from its control 6-rat curve in order that the difference be unlikely due simply to variation in controls? To get an estimate of expected variation in 6-rat control groups, 23 such groups were given 5 per cent of their weight of water by mouth and individually weighed at half-hourly intervals. The mean percentage change in weight of each group at each half-hour was calculated and a grand mean and its standard of all group means at each interval were found. The grand means have been plotted in figure 1 as a heavy solid line and the standard deviations about these grand means have been indicated as a shaded area.

The shaded area includes two-thirds of all expected means in 6-rat control groups measured under these conditions. One curve out of every six may be expected to lie above and one curve below the shaded area. Two curves, one below and one above the shaded area, would not be expected to occur more frequently than 1 out of 36 trials. We took as a working principle that when any curve lay a distance from its control which corresponded to greater than the shaded area in figure 1, a possible significant difference existed and further experiments should be done to increase the number of animals per group. In general, when the space between two curves was less than that corresponding to the extremes of the shaded area in figure 1, such curves were concluded to be insignificantly different and the factor being studied to have little or no effect, not necessarily no effect but an effect, if any, which could probably be disregarded at present in the standardization of technique.

Body weight. Heavy rats were found to lose weight, with or without pituitrin, less rapidly than small rats. This difference in control rats receiving 5 per cent of their weight of water by mouth but no pituitrin is demonstrated in the experiments summarized in table 1. Group A consisted of a total of 36 large rats and group B of 48 small rats and it is ob-

vious that during the first 2 or 3 hours, the small rats lost water at a significantly faster rate than the larger rats. After 3 hours, differences in the rates of water loss became less striking but the differences in the first 3 hours are important because it is over this interval that pituitrin is usually most effective.

In two other groups of rats similar in weight differences to those recorded above, pituitrin was injected in doses of 0.1 international unit per 100 grams' body weight. Over a 5 hour period, pituitrin retained in heavy rats 36 per cent on the average of water lost by similar heavy con-

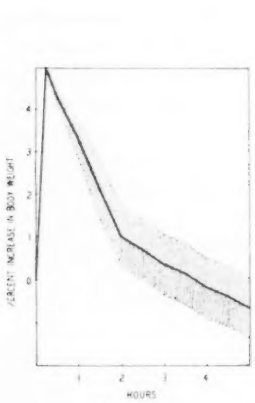

Fig. 1

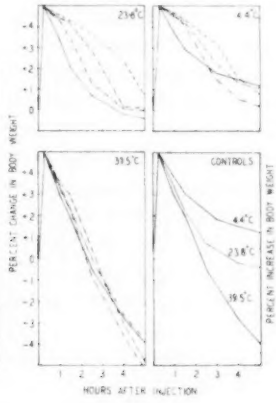

Fig. 2

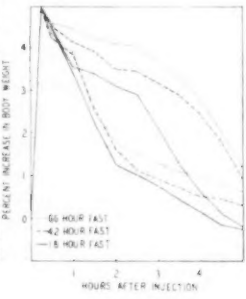

Fig. 3

Fig. 1. Mean changes (solid line) in body weight of 23 groups of 6 albino rats each given by mouth 5 per cent of their weight of water and the standard deviation of the means (shaded area).

Fig. 2. The effect of environmental temperature on changes in the body weight of albino rats given water by mouth without pituitrin (solid lines) and with 0.1 international unit (single dot lines), 0.01 unit (double dot lines) and 0.001 unit (triple dot lines) of pituitrin per 100 grams body weight.

Fig. 3. The effect of various periods of fasting and thirsting on the loss of water given by mouth to rats with (top curves) and without (bottom curves) 1 international unit of pituitrin per 100 grams body weight.

trols not receiving pituitrin. Of the lightweight rats, those injected with pituitrin lost only 27 per cent less water than the controls not receiving pituitrin. The mean difference of 9 per cent had a standard deviation of 4.6 which is just about indicative of a significant difference. In subsequent experiments rats of similar weight were used in all groups to be compared.

Season. The Brunn reaction in frogs varies markedly with season, showing a markedly decreased response in the winter months in this locality (4) and in other respects we have found the body water of frogs to

respond to posterior pituitary extracts differently with seasonal changes. We anticipated a seasonal variation in albino rats but found none. When we averaged percentage changes in body weight of diuretic rats in groups of 30 or more the year around, the mean values were almost identical irrespective of season. Having excluded other suggested causes, seasonal variation in the Brunn reaction was ascribed to some internal factor such as seasonal variation in sexual activity (4). This idea is perhaps born out by the present results since we have not noted in this laboratory any significant seasonal variation in the sexual activity of albino rats as indicated by the ease and success of breeding. We have found rats a little more difficult to breed in January and February but not markedly so.

Environmental temperature. To accentuate any possible effect of environmental temperature, rats were divided into three groups and simultaneously given water by mouth with and without various doses of pituitrin

TABLE 1

The effect of body weight on the loss of water administered by mouth in doses of 5 per cent of body weight to albino rats

VALUE	GROUP	BODY WEIGHT	HOURLY PER CENT CHANGE IN BODY WEIGHT				
			1 Hr.	2 Hrs.	3 Hrs.	4 Hrs.	5 Hrs.
		grams					
Mean	A	250	+3.85	+2.18	+1.07	+0.23	−0.35
Mean	B	165	+3.34	+1.12	+0.54	+0.02	−0.49
Mean difference		85	0.51	1.06	0.53	0.21	0.14
Standard deviation of mean difference		18	0.24	0.26	0.35	0.38	0.35

in one room at a temperature of 4.4°C., in another at 23.8°C., and in a third at 39.5°C. Mean changes in several experiments are shown in figure 2. At room temperature of 23.8°C., the usual type of response was obtained. In a refrigerator room at 4.4°C., rats not receiving pituitrin lost water much less rapidly, the various doses of pituitrin had a less marked and more brief water-retaining effect and in many instances the pituitrin-injected rats had actually lost more water at the end of the experiment than had the controls. At a temperature of 39.5°C., the loss of water was so rapid that pituitrin was ineffective in influencing the changes in body weight. These results indicated that environmental temperature was a factor which required itself to be taken into consideration. In subsequent experiments requiring comparison of data obtained on several different days especially in the summer months when environmental temperature varies here a good deal more than in the winter months in artificially heated

rooms, the factor of temperature was taken into account by including in each day's experiment equal numbers of rats in all groups to be subsequently compared.

Light. Environmental light was investigated because of the well known controversy regarding its reputed effect on the hypophysis and its hormones. Rats were assembled, one group in a dark room in which weighings were made with the aid of a small red light, and another group in the open laboratory. Under these conditions, no appreciable differences were noted in the weight loss of rats with or without injections of pituitrin in various doses.

Air velocity. Rats were assembled in three different rooms in which the temperature and relative humidity were the same but air velocity varied by the use of electric fans. The cooling power of the air in each room was measured by a katathermometer and the dry kata or DK readings were respectively 2.1, 3.1 and 7.9 millicalories per square centimeter of instrument per second. Weight changes in rats assembled in these three rooms with or without pituitrin were identical in corresponding experiments. Weight changes in frogs assembled under similar conditions are markedly affected by air velocity. The difference between frogs and rats is no doubt due to the fact that frogs normally lose a great deal of their water by evaporation through the skin while rats lose very little water in this manner at ordinary temperatures.

Relative humidity. No direct data were obtained on experimental alteration of relative humidity alone but in this connection the following information may be of interest. In the summer, relative humidity readings taken in the laboratory were high, between 50 and 80 per cent saturation, while in the winter with a hot water heating system the relative humidity readings fell to between 10 and 30 per cent saturation. When the results in comparable experiments with animals of the same weight, at the same room temperature, etc., were tabulated and plotted, it was not possible to demonstrate any difference. In this comparison, however, two factors were varying, season and relative humidity and it is just possible that there may have been changes in one which counterbalanced changes due to the other.

Fasting and thirsting. Rats were divided into three groups, one group deprived of food and water for 18 hours, another for 42 hours and a third for 66 hours. Rats in each group were then given water by mouth and varying doses of pituitrin. Mean changes in body weight under these conditions have been plotted in figure 3; the dosage of pituitrin used in the experiments plotted in figure 3 was 1 international unit per 100 grams' body weight. Other doses of pituitrin were also used and the resulting curves were distributed similarly to those shown in figure 3.

The results demonstrated first that a water diuresis could be produced in rats even after several days of thirsting and fasting, a finding which has been previously recorded by Heller and Smirk (6). Pituitrin was more effective in retaining body water in the more dehydrated and fasted rats and this was seen in all experiments. This is an interesting finding since it suggests that the action of pituitrin in rats can be altered by the state of water balance of tissue cells, a conclusion also reached by a different method in experiments reported by Pickford (9).

Salt intake. In the experiment just described, two factors were altered, the water intake and the food intake and probably, though not necessarily, the important part of the food intake was its salt content. Baldes and Smirk (1) recorded that a salt-deficient diet decreases water diuresis and this was confirmed by McCance and Widdowson (7). Unna and Walterskircher (10) demonstrated that pituitrin is markedly diuretic if animals are placed upon a high salt diet. In confirmation of these and other reports, we have found that giving from 50 to 250 mgm. of sodium chloride per 100 grams per day also causes pituitrin to stimulate weight loss instead of retention in rats given water by mouth. These and similar observations are all probably closely related to the original finding of Motzfeldt (8) that pituitrin cannot inhibit a saline diuresis. The phenomenon presumably occurs when the water normally retained by pituitrin is required to dissolve excessive amounts of salt, the urinary excretion of which is normally stimulated by pituitrin.

Chlorbutol in pituitrin. Commercial ampules of pituitrin contain 0.5 per cent of chlorbutol. We have found that chlorbutol itself in doses up to 1 ml. of 0.5 per cent per 100 grams body weight, which corresponds to the chlorbutol in 20 units of Pituitrin Surgical, has no appreciable effect on the loss of water administered to rats.

SUMMARY

When water is administered by mouth to albino rats and loss of body water measured as changes in body weight with and without pituitrin, the following factors affect the reaction. Small rats lose water more rapidly than large rats and pituitrin is less effective in preventing or inhibiting weight loss. Extremes of environmental temperature, either cold or hot, depress the water-retaining effect of pituitrin. Pituitrin retains water more effectively in rats dehydrated and thirsted for a prolonged period. Administered salt decreases the water-retaining effect of pituitrin and may reverse it. Factors which were found to have little or no effect on the reaction of albino rats to pituitrin included season, environmental light, air velocity and relative humdidity.

REFERENCES

(1) Baldes, E. J. and F. H. Smirk. J. Physiol. **82:** 62, 1934.
(2) Boyd, E. M., K. J. Clark and A. E. Smith. This Journal **129:** 645, 1940.
(3) Boyd, E. M. and J. D. Gibson. Proc. Soc. Exper. Biol. and Med. **43:** 572, 1940.
(4) Boyd, E. M., E. G. Mack and A. E. Smith. This Journal **127:** 328, 1939.
(5) Burn, J. H. Quart. J. Pharm. and Pharmacol. **4:** 517, 1931.
(6) Heller, H. and F. H. Smirk. J. Physiol. **76:** 1, 1932.
(7) McCance, R. A. and E. M. Widdowson. J. Physiol. **91:** 222, 1937.
(8) Motzfeldt, J. J. Exper. Med. **25:** 153, 1917.
(9) Pickford, M. J. Physiol. **87:** 291, 1936.
(10) Unna, K. and L. Walterskircher. Arch. exper. Path. und Pharmakol. **181:** 681, 1917.

THE BALANCE OF PHYSICAL FORCES WHICH DETERMINE THE RATE AND DIRECTION OF FLOW OF FLUID THROUGH THE INTESTINAL MUCOSA[1]

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It is the purpose of this paper to present quantitative data which provide further support for a physical hypothesis of intestinal absorption and secretion of fluid.

Previous work of the author (1) has indicated that absorption of fluid from the intestine may be effected by the energy of colloid osmotic forces exerted by proteins in the tissue fluids of the villi; for it was found, in the case of each of a series of animals, that the negative intra-intestinal pressure required to abolish absorption was equal to the colloid osmotic pressure of lymph from the lacteals of the same loop of gut. The author has suggested (2) that secretion of fluid by the bowel may result chiefly from the operation of simple physical mechanisms. Elevation of tissue pressure in the villi may result in ultrafiltration of tissue fluids through the epithelial membrane into the lumen of the gut. During secretion the villi are observed to be swollen, often to twice their resting diameter, and this appearance leads one to believe that tissue pressure becomes elevated as the result of filtration of fluid from the local capillaries. As a matter of fact, these vessels are congested, and the tonic contraction of the *muscularis mucosae* (which accompanies the secretion resulting from pilocaropine or from irritation of the mucosa) appears to offer obstruction to the drainage both of venous blood and of lymph. Although attempts have been made to measure tissue pressure in the mucosa by methods employed successfully in other tissues (3) local bleeding has always rendered these studies valueless. The present paper offers another, indirect, approach which seems to solve the problem; for the value of tissue pressure may be estimated in terms of capillary and osmotic pressures, under specified conditions.

The physical hypothesis of fluid transfer may be expressed in the form of the equation:

$$\frac{dW}{dt} = k(TP - OP_t - IP) \tag{1}$$

[1] This work was aided by a Fluid Research Fund of the Rockefeller Foundation.

In this formula, $\frac{dW}{dt}$, the rate of flow of fluid through the gut wall, is positive when fluid is secreted and negative during absorption. TP is the hydrostatic pressure and OP_t is the colloid osmotic pressure of the tissue fluids of the villi; IP is the intra-intestinal pressure; and k is the constant of proportionality. Since k varies with the area and permeability of the intestinal mucosa (factors which cannot be accurately measured) comparison of data from various animals is facilitated if the pressures can be so adjusted that neither absorption nor secretion occurs. Under these conditions $\frac{dW}{dt} = 0$, and the formula becomes:

$$TP = OP_t + IP \tag{2}$$

When the gut is in the absorbing state—the villi being slender and the blood flow sluggish—it may be assumed that TP is close to atmospheric pressure. When $TP = 0$ the equation reduces to the form:

$$OP_t = -IP \tag{3}$$

On the assumption that OP_t is the same as the colloid osmotic pressure of lymph from the lacteals, form (3) of the equation expresses the actual conditions required to balance the forces of secretion and absorption when the gut is in the absorbing state (1).

Except for the special case when secretion results from the application of excessive negative pressure to the contents of a loop of bowel (4), the mucosa of which is in the absorbing state, one finds that the villi and the whole thickness of the mucosa are swollen and turgid with fluid whenever secretion occurs. It may be assumed that the tissue pressure, TP, has a positive value when secretion is produced by mechanical or chemical stimulation of the mucosa, by cutting the mesenteric nerves, by intravenous injection of large amounts of saline, by injections of pilocarpine, or by procedures which elevate the portal venous pressure to a sufficient degree. Consequently the value of TP must be considered in any attempt at quantitative validation of the physical hypothesis of fluid transfer which includes a study of normal secretion. Since the value of TP cannot be obtained by direct measurement, it is fortunate that this variable can be eliminated from our equation by the substitution of equivalent quantities. It is evident that the physical hypothesis involves the factor of capillary filtration—the process by which fluid is supplied during secretion—and it must be assumed that, when intestinal absorption and secretion are balanced, capillary-absorbing and capillary-filtering forces are also in balance, provided fluid cannot escape from the villi by the lymphatic channels. Under these conditions:

$$CP - TP = OP_b - OP_t \tag{4}$$

In this equation CP is the mean capillary pressure, TP is the tissue pressure, $OP_b - OP_t$ represents the effective colloid osmotic pressure: the difference between the total colloid osmotic pressure of the blood and the osmotic pressure of proteins in the tissue fluids. When the value of TP from equation (2) is substituted in equation (4) we obtain:

$$CP - IP = OP_b \tag{5}$$

Equation (5) contains variables IP and OP_b which are easily measured, but since the capillary pressure in the villous circulation cannot be determined directly it is necessary to estimate CP indirectly. We have caused CP to vary by producing various degrees of congestion of the mesenteric veins. When properly applied to the mesenteric vessels, the crescent-

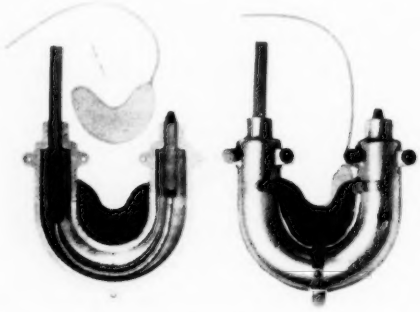

Fig. 1. Apparatus employed for congesting mesenteric vessels. Originally designed as a plethysmograph, the device was used, during this research, merely to provide support for parallel metal plates, between which were placed the mesentery of the isolated loop and the soft rubber bag used to produce congestion. The helical coil of aluminum wire, attached to its rubber-covered glass cannulae, is shown at the left. This device is placed inside the loop of gut, the ends of which are tied tightly over the cannulae.

shaped rubber bag, pictured in figure 1, may be blown up to compress the veins and lymphatics. Filtration of fluid from villous capillaries is doubtless accelerated by the resulting elevation of capillary pressure. The accumulating tissue fluid eventually swells the villi because its escape by way of the mesenteric lacteals is cut off at the point of application of the pressure bag.

It was found experimentally that, corresponding to any given intra-intestinal pressure—pressures slightly below atmospheric were employed throughout—there was a definite mesenteric venous pressure, measured by a water manometer connected to the rubber bag system, which exactly[2]

[2] The greatest error in determining each of the pressures which were measured (mesenteric venous, intra-intestinal, and colloid osmotic pressures) does not exceed

balanced the forces tending to cause absorption of fluid. Since venous pressure, during passive congestion, is probably not much lower than mean capillary pressure, it is not surprising to find, when the values of mesenteric venous pressure are substituted for CP in equation (5), that the equation actually expresses the conditions for the balancing of fluid-absorbing and fluid-secreting forces of the intestine. The graph of the data, shown in figure 2, indicates that fluid transfer through the gut wall will cease when mesenteric venous pressure and intra-intestinal pressure are so adjusted that their difference is equal to the total colloid osmotic pressure of the animal's blood serum. The line drawn at a slope of 45 degrees from the origin is the locus of points representing exact equality of $CP - IP$ and OP_b. It will be noted that the experimental points arrange themselves fairly accurately along the theoretical line.

The data were obtained from experiments on 10 dogs, anesthetized with sodium barbital (0.25 gram/kgm., injected intravenously). Single determinations were made on 7 animals. The eighth dog received an intravenous injection of 100 cc. of 30 per cent acacia solution, which elevated the osmotic pressure of the blood from its original value of 26 cm. of water to the high level of 42 cm. of water. The corresponding value of $CP - IP$ underwent a change of the order to be expected from the physical hypothesis of fluid transfer as expressed in equation (5). Another dog was bled until the osmotic pressure of its blood fell from 27 to 20 cm. of water. The tenth dog was bled four separate times, the osmotic pressure falling from the original value of 29 cm. to successive values of 24, 19, 14 and 10.5 cm. of water. In order to maintain the balance between absorbing and secreting forces it was necessary to diminish the congesting pressure at each stage, as indicated in the figure.

Intra-intestinal pressure was maintained, during these experiments, at from -3 to -5 cm. of water. The helical coil of aluminum wire, previously described (4) and pictured in figure 1, prevented collapse of the gut walls when internal pressure was reduced below atmospheric. The congesting pressures, required to balance absorbing and secreting forces, varied from 8 to 40 cm. of water. In one experiment intra-intestinal pressure was set at successive levels of -2, -6 and -11 cm. of water. The corresponding values of mesenteric venous pressure required to prevent fluid transfer in either direction were 24, 20 and 14 cm. of water, respectively. The values of $CP - IP$ were therefore 26, 26 and 25 cm. of water respectively: a fairly good check on the validity of the assumption that variations in venous pressure and intra-intestinal pressure are equally

1.0 cm. of water. A change of less than 0.5 cm. of water in any of these pressures will produce a readily detectable change (0.01 cc. per minute or greater) in the rate of absorption or secretion.

effective in influencing the transfer of fluid, at the point of balance between secretion and absorption.

It has been observed that during congestion of the mesenteric veins and lacteals, the whole thickness of the gut wall becomes swollen, and fluid weeps constantly from the peritoneal surface of the loop. This fluid loss

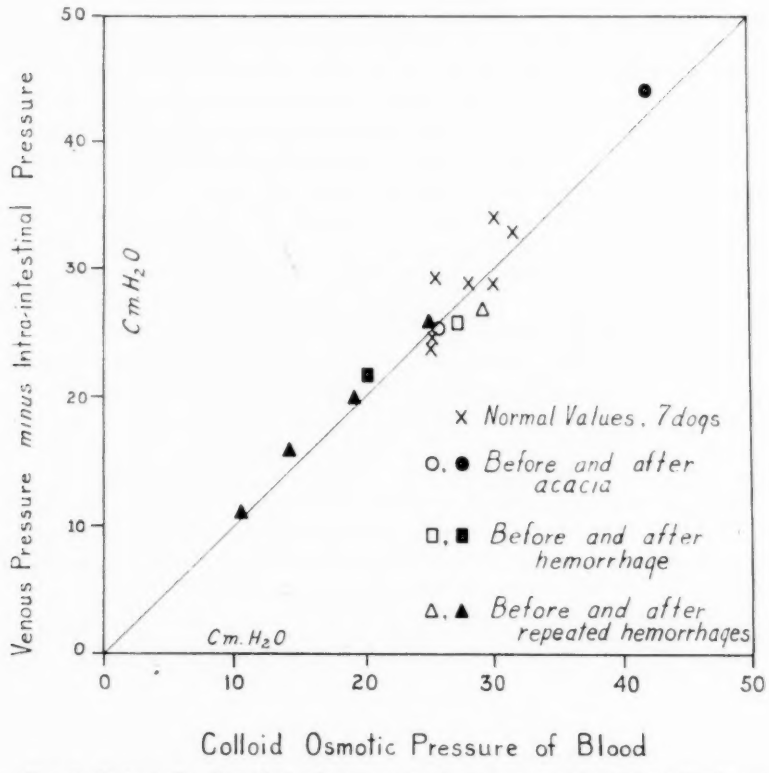

Fig. 2. Data indicating that when mesenteric venous and intra-intestinal pressures are adjusted to balance fluid-absorbing and secreting forces the difference of these values is approximately equal to the colloid osmotic pressure of the blood, over a wide range of values of the latter. The diagonal line is the locus of points representing exact equality of these variables, as demanded by theory.

presents possibilities for error in the analysis, for it has been tacitly assumed, in the discussion of the mathematical physics of the filtering-absorbing system, that the whole mechanism is enclosed in elastic, non-leaking walls. Consequently when we examine the graph of figure 2 it is not perhaps surprising that more points are found to lie above the theo-

retical line than below it. It seems evident that, in a slightly leaky system, capillary pressure must be maintained above the true point of equilibrium in order to provide for filtration at a rate equal to the losses from leakage. Most of this leakage, to be sure, may take place from the capillaries of the muscular coats and peritoneal surface, but it would be logical to believe that fluid can also escape by filtration from thin-walled lacteals as they penetrate the sub-mucosa and enter the muscularis. During the normal process of secretion such leakage presumably does not occur, since the veins and central lacteal of each villus are constricted at the base of the villus by the tonic contraction of the *muscularis mucosae*.

The error of estimating capillary pressure from venous pressure will tend to make the experimental points of figure 2 fall below the line. It is possible that this error in the negative direction may have become combined, in some instances, with the positive error mentioned in the preceding paragraph to give, by chance, the almost exact concordance of observed and theoretical values represented by the points which lie closest to the line.

Relation of intra-intestinal, congesting, and osmotic pressures to the rate of secretion of intestinal juice. If, at any constant value of intra-intestinal pressure, the congestion of the mesenteric veins and lymphatics is increased beyond the point of fluid balance, secretion will occur at a rate which attains a constant value, the magnitude of which is determined by the congesting pressure. A loop of bowel 20 cm. long may be caused, in this manner, to secrete fluid at a rate of 0.5 to 1.0 cc. per minute without becoming damaged; but at congesting pressures above 40 to 60 cm. of water, hemorrhage from the mucosa may complicate the experiment. Usually the effects of congestion are completely reversible; thus, upon release of the congesting pressure, the gut, within a few minutes, resumes absorption of fluid at precisely the rate observed during the period preceding the congestion.

If the congesting pressure is elevated in successive steps above the point of fluid balance, fluid is secreted at rates which increase geometrically rather than in direct ratio to the increments of venous pressure. Changes in intra-intestinal pressure at constant congesting pressure produce nearly proportional changes in the rate of secretion or absorption, but the constant of proportionality increases as the congesting pressure is increased. Stated in another way, the relation of fluid flow to intra-intestinal pressure is approximately linear, but the slope of the line increases geometrically as congesting pressure increases. It is possible that these deviations from proportionality are due to a progressive increase in the surface and a diminishing thickness of the epithelial membrane of the villi as they become progressively more swollen by increasing grades of congestion. There are other possibilities, but this explanation seems most

probable, for visual observation of the villi, which was made possible by the use of a special glass-covered chamber previously described (2), showed definitely that the villi did become swollen whenever the venous outflow from the loop was partially obstructed, and the degree of swelling paralleled the degree of venous congestion.

It has not seemed necessary to make special tests of enzymatic action to determine whether the juice put out by the mucosa as the result of venous congestion is normal succus entericus. In the first place, we are interested primarily in the exchange of fluid and not in its composition with respect to solutes. In the second place, Nasset and Parry (5) have found that the fluid which may be "sucked" into the gut by negative intra-intestinal pressure possesses the properties of normal intestinal juice; and there is no reason to believe that the "congestion juice" should differ from "suction juice" in any significant manner. The fluid which we have obtained was opalescent, and it contained no trace of blood.

As might be expected from the discussion so far, changes in colloid osmotic pressure of the blood are reflected in corresponding variations in the absorbing force of the gut, and in inverse changes in the rate of secretion. Thus, if an animal is bled and the volume of blood removed is replaced by saline, or if excessive amounts of saline are injected into the normal animal, without previous hemorrhage, secretion will start within a minute or two in a loop of bowel the intra-intestinal and congesting pressures of which have been previously adjusted to the point of fluid balance. In the case of the dog which suffered a single hemorrhage (200 cc.) the absorbing force, as measured by the intra-intestinal pressure necessary to balance the flow of fluid without venous congestion, fell from its original value of 19 cm. to 9 cm. of water.

Discussion. Whether physical forces are or are not entirely responsible for the transfer of fluid through the intestinal mucosa, it is clear that variations in capillary, colloid osmotic, tissue and intra-intestinal pressures, each and severally, produce profound effects on the absorption and secretion of water. The fact that venous and lymphatic congestion of sufficient grade will invariably stop the absorption of water and that more severe degrees of congestion will cause significantly rapid losses of fluid into the lumen of the gut as well as into the peritoneal cavity are facts which are of importance for clinical medicine. The degree of congestion required to balance the forces of absorption—35 cm. of water is an average normal value of the colloid osmotic pressure of the blood of man—is probably not outside the range of values of venous pressure observed clinically in patients suffering from congestive heart failure, constrictive pericarditis, portal cirrhosis, etc. It would be well to point out, however, that values of venous or intra-intestinal pressure may not be used, in the equations which we have derived, unless these pressures are measured in relation to intra-abdominal pressure as the zero point.

The work of Herrin and Meek (6) on intestinal obstruction showed that distention of the bowel sets up a copious secretion resulting in important losses of fluid and salts from the body. Previous experiments of our own (unpublished) indicate that distention results in venous obstruction, and therefore it may be presumed that the secretion is due to the elevation of capillary and tissue pressures.

We have found that dogs which are very deeply anesthetized with barbital (0.35 gram per kilo) often show enlarged spleens, engorged mesenteric veins and portal pressures up to 45 cm. of water. When loops of bowel are isolated for study in such animals they invariably secrete continuously until the venous pressure is lowered to a normal level by the administration of a full dose of atropine, whereupon absorption only rarely fails to set in. The action of atropine to facilitate absorption of fluid has been noted by others (7). It is possible that the effect just described is due to some action of the drug on the hepatic sluice mechanism; but atropine also relaxes the outer gut musculature, an effect which should facilitate absorption in cases in which the tone of these muscles causes local venous congestion.

Objections to the physical hypothesis of intestinal fluid transfer have been raised by several writers. Some have insisted that absorption and secretion of fluid occur simultaneously; that changes of volume of solutions placed in the gut therefore represent the resultant effects of the two processes; and that, consequently, attempts to measure the rates of fluid absorption and secretion separately are doomed to failure. This pessimistic view appears to be based on the preconceived idea—no longer tenable—that living cells are unable to transport separate ions or molecules, but must move solutions en masse. The fact that water can be absorbed while salts and other blood constituents and enzymes are passing outward into the contents of the gut is no more difficult to explain by well known laws of diffusion than is absorption of tissue fluids through the walls of a blood capillary at the same time that a dye is diffusing out of the capillary into the tissue spaces.

The fact that the gut can absorb water from a hypertonic solution of NaCl has been considered as proof of the existence of special absorbing forces capable of operating against several atmospheres of osmotic pressure. But the laws of osmotic pressure tell us nothing whatsoever about the initial direction of movement of water from one solution to another except when the membrane separating them is absolutely impermeable to all solutes. Ions or molecules which are diffusing rapidly through a membrane may be expected to "carry water with them" in what appears to be the wrong direction. It was shown by Lazarus-Barlow in 1896 (8) that ox serum would absorb water from a 2 per cent NaCl solution across an artificial membrane which was permeable to the salt but impermeable to the serum proteins. Most physiologists realize that red blood cor-

puscles are quickly hemolyzed by hyperosmotic solutions of urea in water. Thermodynamic relations of this type of "anomalous" osmosis have been discussed by Shreinemakers (9).

Colloid osmotic forces have for years been considered inadequate to account for the absorption of water from blood serum or solutions of acacia. However, as Rabinovitch (8) and the author (unpublished) have shown, serum and acacia are absorbed at precisely the same rate as isotonic saline. This very exact equality of the absorption rates persists even at negative intra-intestinal pressures, and absorption of serum is retarded and eventually stopped by increasing degrees of negative pressure in the same manner and to the same extent as absorption of saline is affected by these relatively small changes in the applied force. These experiments, so far as they go, merely indicate that the proteins and acacia are, in some unknown manner, rendered completely incapable of exerting any colloid osmotic pressure, when they are brought in contact with the surface of the mucosa. The problem invites further study.

It has been suggested that the anesthesia or the negative intra-intestinal pressure employed in our experiments may abolish some of the special or selective absorbing or secretory forces of the gut, leaving unaffected those simple physical forces which the author has measured in previous studies; but the fact that "chloride impoverishment" (10) and absorption of serum—phenomena which involve special selective activity—have been shown to persist in our animals under the conditions prevailing in all of our experiments, would seem to rule out such objections to the physical hypothesis.

The author is well aware of the fact that the intestinal juice is not in equilibrium with the blood, so far as the various solute concentrations, pH, etc., are concerned (11, 12). It is clear that the mucosa performs osmotic work upon dissolved materials; but neither these phenomena nor the fact that the mucosa secretes mucus and enzymes need have any more bearing on absorption or secretion of water than the secretion of ammonia by the kidney has upon the process of glomerular filtration. There are no data which compel the belief that water is ever transferred by the gut against a true osmotic gradient.

It is urged, finally, that the scientific principle of economy of hypothesis favors the unitary and relatively simple theory proposed by the author to account for the transfer of fluid through the gut wall.

The remarkable experiments of Königes and Ottó (13), who find that central lacteal pressure in the villi of cats may attain values (24 to 25 mm. Hg.) considerably in excess of the colloid osmotic pressure of intestinal lymph (10 to 11 mm. Hg.), lend direct support to the physical hypothesis of intestinal secretion, and indicate that future study of the problems of fluid transfer may, after all, involve the use of direct micro-

pipette determinations of the capillary and tissue fluid pressures within the villi.

SUMMARY

1. Moderate congestion of the mesenteric veins and lymphatics produces slowing of the rate of absorption of isotonic saline from isolated loops of the small intestine. At a definite congesting pressure absorbing and secreting forces may be exactly balanced. Higher grades of congestion produce secretion of intestinal juice, the rates of secretion increasing geometrically as mesenteric venous pressure is increased above the value required to abolish absorption.

2. Analysis of the physical hypothesis which has been proposed to account for intestinal absorption and secretion of fluid, indicates that absorbing and secreting forces should be neutralized when the difference between mean capillary pressure in the villi and intra-intestinal pressure is equal to the colloid osmotic pressure of the animal's blood plasma. When venous pressure is used as the measure of capillary pressure the equation fits the data obtained experimentally.

3. Some objections, commonly raised against physical hypotheses of intestinal transfer of fluids, are discussed.

REFERENCES

(1) Wells, H. S. This Journal **101:** 434, 1932.
(2) Wells, H. S. and R. G. Johnson. This Journal **109:** 387, 1934.
(3) Wells, H. S., J. B. Youmans and D. G. Miller, Jr. J. Clin. Investigation **17:** 489, 1938.
(4) Wells, H. S. This Journal **99:** 209, 1931.
(5) Nasset, E. S. and A. A. Parry. This Journal **109:** 614, 1934.
(6) Herrin, R. C. and W. J. Meek. Arch. Int. Med. **51:** 152, 1933.
(7) Rabinovitch, J. This Journal **82:** 279, 1927.
(8) Lazarus-Barlow, W. S. J. Physiol. **20:** 145, 1896.
(9) Shreinemakers, F. A. H. J. Gen. Physiol. **11:** 701, 1928; **12:** 555, 1929.
(10) Ingraham, R. C. and M. B. Visscher. This Journal **114:** 676, 1936.
(11) DeBeer, E. J., E. G. Johnston and D. W. Wilson. J. Biol. Chem. **108:** 113, 1935.
(12) Robinson, C. S. J. Biol. Chem. **108:** 403, 1935.
(13) Königes, H. G. and M. Ottó. Quart. J. Exper. Physiol. **26:** 319, 1937.